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(57) Abstract

The invention involves multicomponent complexes, which use useful in analysis of T cells. The complexes contain at least first and second binding partners, which bind to each other. The second binding partner engages a plurality of immune complexes, which comprise an NHC molecule, a $\beta2$ microglobulin molecule, and a peptide. Preferably, there are at least four of these immune complexes per multicomponent complex. These can be used to determine or to isolate cytolytic T cells.

ISOLATED MULTIMERIC COMPLEXES USEFUL IN ANALYSIS OF T CELLS, PEPTIDES USEFUL IN MAKING THE COMPLEXES, AND USES THEREOF

RELATED APPLICATION

5   This application is a continuation in part of Serial No. 09/049,850, filed on March 27,

1998, and incorporated by reference in its entirety.

FIELD OF THE INVENTION

This invention relates to multicomponent complexes which are useful in analysis of T

cell populations.

RELATED ART

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Certain aspects of this invention will be found in published materials involving some of

the inventors. See Dunbar et al., "Characterization of Human Cytotoxic T Lymphocyte (CTL)

responses To Tumor Antigens Using Fluorogenic MHC Class I/Peptide Complexes", Tumor

Immunology 92 Suppl. Dec. 1997, reporting abstract 3.3 of the 5th Annual Congress of the BSI.

15   Also see Dunbar et al., "Direct isolation, phenotyping and cloning of law-frequency antigen

specific cytotoxic T lymphocytes from peripheral blood", Curr. Biol. (in press), but possibly

available via Internet on March 16, 1998.

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BACKGROUND AND PRIOR ART

The study of the recognition or lack of recognition of cancer cells by a host organism has proceeded in many different directions. Understanding of the field presumes some understanding of both basic immunology and oncology.

5   Early research on mouse tumors revealed that these displayed molecules which led to rejection of tumor cells when transplanted into syngeneic animals. These molecules are "recognized" by T-cells in the recipient animal, and provoke a cytolytic T-cell response with lysis of the transplanted cells. This evidence was first obtained with tumors induced in vitro by chemical carcinogens, such as methylcholanthrene. The antigens expressed by the tumors and

10   which elicited the T-cell response were found to be different for each tumor. See Prehn, et al., J. Natl. Canc. Inst. 18: 769-778 (1957); Klein et al., Cancer Res. 20: 1561-1572 (1960); Gross, Cancer Res. 3: 326-333 (1943), Basombrio, Cancer Res. 30: 2458-2462 (1970) for general teachings on inducing tumors with chemical carcinogens and differences in cell surface antigens. This class of antigens has come to be known as "tumor specific transplantation antigens" or

15   "TSTAs". Following the observation of the presentation of such antigens when induced by chemical carcinogens, similar results were obtained when tumors were induced in vitro via ultraviolet radiation. See Kripke, J. Natl. Canc. Inst. 53: 333-1336 (1974).

While T-cell mediated immune responses were observed for the types of tumor described supra, spontaneous tumors were thought to be generally non-immunogenic. These were

20   therefore believed not to present antigens which provoked a response to the tumor in the tumor carrying subject. See Hewitt, et al., Brit. J. Cancer 33: 241-259 (1976).

The family of tum⁻ antigen presenting cell lines are immunogenic variants obtained by mutagenesis of mouse tumor cells or cell lines, as described by Boon et al., J. Exp. Med. 152:

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1184-1193 (1980), the disclosure of which is incorporated by reference. To elaborate, tum⁻

antigens are obtained by mutating tumor cells which do not generate an immune response in

syngeneic mice and will form tumors (i.e., "tum⁺" cells). When these tum⁺ cells are

mutagenized, they are rejected by syngeneic mice, and fail to form tumors (thus "tum⁻"). See

Boon et al., Proc. Natl. Acad. Sci. USA 74: 272 (1977), the disclosure of which is incorporated

by reference. Many tumor types have been shown to exhibit this phenomenon. See, e.g., Frost

et al., Cancer Res. 43: 125 (1983).

It appears that tum⁻ variants fail to form progressive tumors because they initiate an

immune rejection process. The evidence in favor of this hypothesis includes the ability of "tum⁻"

variants of tumors, i.e., those which do not normally form tumors, to do so in mice with immune

systems suppressed by sublethal irradiation, Van Pel et al., Proc. Natl. Acad. Sci. USA 76: 5282-

5285 (1979); and the observation that intraperitoneally injected tum⁻ cells of mastocytoma P815

multiply exponentially for 12-15 days, and then are eliminated in only a few days in the midst

of an influx of lymphocytes and macrophages (Uyttenhove et al., J. Exp. Med. 152: 1175-1183

(1980)). Further evidence includes the observation that mice acquire an immune memory which

permits them to resist subsequent challenge to the same tum⁻ variant, even when

immunosuppressive amounts of radiation are administered with the following challenge of cells

(Boon et al., Proc. Natl, Acad. Sci. USA 74: 272-275 (1977); Van Pel et al., supra; Uyttenhove

et al., supra). Later research found that when spontaneous tumors were subjected to

mutagenesis, immunogenic variants were produced which did generate a response. Indeed, these

variants were able to elicit an immune protective response against the original tumor. See Van

Pel et al., J. Exp. Med. 157: 1992-2001 (1983). Thus, it has been shown that it is possible to

elicit presentation of a so-called "tumor rejection antigen" in a tumor which is a target for a

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syngeneic rejection response. Similar results have been obtained when foreign genes have been

transfected into spontaneous tumors. See Fearon et al., Cancer Res. 48: 2975-1980 (1988) in this

regard.

A class of antigens has been recognized which are presented on the surface of tumor cells

5   and are recognized by cytolytic T cells, leading to lysis. This class of antigens will be referred

to as "tumor rejection antigens" or "TRAs" hereafter. TRAs may or may not elicit antibody

responses. The extent to which these antigens have been studied, has been via cytolytic T cell

characterization studies, in vitro i.e., the study of the identification of the antigen by a particular

cytolytic T cell ("CTL" hereafter) subset. The subset proliferates upon recognition of the

10   presented tumor rejection antigen, and the cells presenting the tumor rejection antigens are lysed.

Characterization studies have identified CTL clones which specifically lyse cells expressing the

tumor rejection antigens. Examples of this work may be found in Levy et al., Adv. Cancer Res.

24: 1-59 (1977); Boon et al., J. Exp. Med. 152: 1184-1193 (1980); Brunner et al., J. Immunol.

124: 1627-1634 (1980); Maryanski et al., Eur. J. Immunol. 124: 1627-1634 (1980); Maryanski

15   et al., Eur. J. Immunol. 12: 406-412 (1982); Palladino et al., Canc. Res. 47: 5074-5079 (1987).

This type of analysis is required for other types of antigens recognized by CTLs, including minor

histocompatibility antigens, the male specific H-Y antigens, and the class of antigens referred

to as "tum⁻" antigens, and discussed herein.

A tumor exemplary of the subject matter described supra is known as P815. See DePlaen

20   et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988); Szikora et al., EMBO J 9: 1041-1050

(1990), and Sibille et al., J. Exp. Med. 172: 35-45 (1990), the disclosures of which are

incorporated by reference. The P815 tumor is a mastocytoma, induced in a DBA/2 mouse with

methylcholanthrene and cultured as both an in vitro tumor and a cell line. The P815 line has

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generated many tum⁻ variants following mutagenesis, including variants referred to as P91A
(DePlaen, supra), 35B (Szikora, supra), and P198 (Sibille, supra). In contrast to tumor rejection
antigens - and this is a key distinction - the tum⁻ antigens are only present after the tumor cells
are mutagenized. Tumor rejection antigens are present on cells of a given tumor without

5   mutagenesis. Hence, with reference to the literature, a cell line can be tum⁺, such as the line
referred to as "P1", and can be provoked to produce tum⁻ variants. Since the tum⁻ phenotype
differs from that of the parent cell line, one expects a difference in the DNA of tum⁻ cell lines
as compared to their tum⁺ parental lines, and this difference can be exploited to locate the gene
of interest in tum⁻ cells. As a result, it was found that genes of tum⁻ variants such as P91A, 35B

10   and P198 differ from their normal alleles by point mutations in the coding regions of the gene.
See Szikora and Sibille, supra, and Lurquin et al., Cell 58: 293-303 (1989). This has proved not
to be the case with the TRAs of this invention. These papers also demonstrated that peptides
derived from the tum⁻ antigen are presented by H-2^d Class I molecules for recognition by CTLs.
P91A is presented by L^d, P35 by D^d and P198 by K^d.

15   PCT application PCT/US92/04354, filed on May 22, 1992 assigned to the same assignee
as the subject application, teaches a family of human tumor rejection antigen precursor coding
genes, referred to as the MAGE family. Several of these genes are also discussed in van der
Bruggen et al., Science 254: 1643 (1991). It is now clear that the various genes of the MAGE
family are expressed in tumor cells, and can serve as markers for the diagnosis of such tumors,

20   as well as for other purposes discussed therein. See also Traversari et al., Immunogenetics 35:
145 (1992); van der Bruggen et al., Science 254: 1643 (1991) and De Plaen, et al.,
Immunogenetics 40: 360 (1994). The mechanism by which a protein is processed and presented
on a cell surface has now been fairly well documented. A cursory review of the development

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of the field may be found in Barinaga, "Getting Some 'Backbone': How MHC Binds Peptides", Science 257: 880 (1992); also, see Fremont et al., Science 257: 919 (1992); Matsumura et al., Science 257: 927 (1992); Engelhard, Ann. Rev. Immunol 12:181-207 (1994); Madden, et al., Cell 75:693-708 (1993); Ramensee, et al., Ann. Rev. Immunol 11:213-244 (1993); German, Cell

5   76: 287-299 (1994). These papers generally point to a requirement that the peptide which binds to an MHC/HLA molecule be nine amino acids long (a "nonapeptide"), and to the importance of the second and ninth residues of the nonapeptide. For H-2K^b, the anchor residues are positions 5 and 8 of an octamer, for H-2D^b, they are positions 5 and 9 of a nonapeptide while the anchor residues for HLA-A1 are positions 3 and 9 of a nonamer. Generally, for HLA molecules,

10   positions 2 and 9 are anchors.

Studies on the MAGE family of genes and many other TRAPs have now revealed that these are processed to peptides which complex with HLA molecules, leading to lysis of the cells presenting these by cytolytic T cells ("CTLs").

Research presented in, e.g., U.S. Patent No. 5,405,940 filed August 31, 1992, and in U.S.

15   Patent No. 5,571,711, as well as many other issued U.S. patents found that when comparing homologous regions of various MAGE genes to the region of the MAGE-1 gene coding for the relevant nonapeptide, there is a great deal of homology, and identification of a family of nonapeptides, all of which have the same N-terminal and C-terminal amino acids. These nonapeptides are useful for various purposes including their use as immunogens, either alone or

20   coupled to carrier peptides. Nonapeptides are of sufficient size to constitute an antigenic epitope, and the antibodies generated thereto were described as being useful for identifying the nonapeptide, either as it exists alone, or as part of a larger polypeptide.

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The preceding survey of the relevant literature shows that various peptides, usually eight, nine, or ten amino acids in length, complex with MHC molecules and present targets for recognition by cytolytic T cells. A great deal of study has been carried out on melanoma, and melanoma antigens which are recognized by cytolytic T cells are now divided into three broad categories. The first, which includes many of the antigens discussed, supra, (e.g., MAGE), are expressed in some melanomas, as well as other tumor types, and normal testis and placenta. The antigens are the expression product of normal genes which are usually silent in normal tissues.

A second family of melanoma antigens includes antigens which are derived from mutant forms of normal proteins. Examples of this family are MUM-1 (Coulie, et al., Proc. Natl. Acad. Sci. USA 92:7976-7980(1955)); CDK4 (Wölfel, et al., Science 269:1281-1284(1955)); Bcatenin (Robbins, et al., J. Exp. Med. 183:1185-1192 (1996)); and HLA-A2 (Brandel, et al., J. Exp. Med. 183:2501-2508 (1996)). A third category, also discussed, supra, includes the differentiation antigens which are expressed by both melanoma and melanocytes. Exemplary are tyrosinase gp100, gp75, and Melan A/Mart-1. See U.S. Patent No. 5,620,886 incorporated by reference, with respect to Melan-A. See Wölfel, et al., Eur. J. Immunol. 24: 759 (1994) and Brichard, et al., Eur. J. Immunol. 26: 224 (1996) for tyrosinase; Kang, et al., J. Immunol. 155: 1343 (1995); Cox, et al., Science 264: 716 (1994); Kawakami, et al., J. Immunol. 154: 3961 (1995) for gp 100; Wang, et al., J. Exp. Med. 183: 1131 (1996) for gp 75.

Cytolytic T cells ("CTLs" hereafter) have been identified in peripheral blood lymphocytes, and tumor infiltrating lymphocytes, of melanoma patients such as those who are HLA-A*0201 positive. See Kawakami, et al, Proc. Natl. Acad. Sci. USA 91:3515 (1994); Coulie, et al, J. Exp. Med. 180:35 (1994). When ten HLA-A*0201 restricted Melan-A specific CTLs were derived from different patients were tested, nine of them were found to recognize and

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react with the peptide Ala Ala Gly Ile Gly Ile Leu Thr Val, (SEQ ID NO:1), which consists of

amino acids 27-35 of Melan-A. (Kawakami, et al, J. Exp. Med 180:347-352 (1994)). Rivoltini,

et al, J. Immunol 154:2257 (1995), showed that Melan-A specific CTLs could be induced by

stimulating PBLs from HLA-A˙0201 positive normal donors, and melanoma patients, using SEQ

5   ID NO:1. The strength of this response has led to SEQ ID NO:1 being proposed as a target for

vaccine development. Further research has shown that a decapeptide, i.e.,

        Glu Ala Ala Gly Ile Gly Ile Leu Thr Val

(SEQ ID NO:2), is actually a better target than SEQ ID NO:1. See U.S. Patent Application

Serial No. 08/880,963, filed June 23 1997 and incorporated by references.

10   One difficulty in the area of cancer immunology is a lack of reliable protocols which can

be used to identify and to quantify in vivo cytolytic T lymphocyte responses. As a result, it is

difficult to characterize immune response, and to monitor vaccine trials. The invention described

hereafter was developed to address these, and other issues in the field. It has been found that

analysis of cytolytic T cells is greatly facilitated by the use of complexes containing a plurality

15   of T cell targets. More specifically, the invention relies on the known avidity of two binding

partners, such as avidin or streptavidin and biotin for each other. It is well known that every

molecule of avidin/streptavidin can bind to four biotin molecules. The invention involves

constructs where the avidin/streptavidin-biotin system is used to form complexes containing

multiple targets for cytolytic T cells, i.e., a plurality of immune complexes which comprise an

20   MHC molecule, such as an HLA molecule, a ß2 microglobulin, and a peptide which binds to the

HLA molecule. The complex is labelled, and can be used to isolate, or to determine, cytolytic

T cells of interest in a sample.

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How these and other features of the invention are achieved will be seen in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a correlation between frequency of cells positive for particular peptide containing

5   tetramers and cytotoxicity.

Figure 2 shows that CD8$^+$ cells, isolated from bulk cultures and stimulated and expanded without antigen had high tumoricidal activity.

Figure 3 presents data on determination of relative activity of peptides.

Figure 4 sets forth data showing that complexes of HLA-A2 and SEQ ID NO: 4 are more stable

10   than complexes of HLA-A2 and SEQ ID NO: 1.

Figure 5 presents data contributing to analysis of both polyclonal and monoclonal T cell populations specific for tetramers of HLA-A* 0201 and SEQ ID NO: 4.

Figure 6 presents phenotype data for CD8$^+$ cells specific for particular peptide containing tetramers.

15   Figure 7A-7C, inclusive, depict the results of experiments designed to compare fine specificities of unsorted TILN populations.

Figure 8 summarizes data on stimulation of cytokine production by polyclonal and monoclonal cell populations.

Figure 9 summarizes results of experiments designed to test PBL activity in the presence of NK

20   receptor specific monoclonal antibodies.

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Example 1

In order to make the desired tetramers, it was first necessary to prepare constructs which would encode modified HLA-A*0201 molecules. To do this, total RNA was extracted from HLA-A*0201 positive cells, and HLA-A*0201 was then cloned, using specific primers for the molecule, and reverse transcription polymerase chain reaction (RT-PCR). Altman et al., Science 274: 94-96 (October 4, 1996) but with a new 3' primer, i.e. 5'-GCAGGATCCCGGCTCCCATCCTCA GGGTGAGGGGC-3' (SEQ ID NO: 48) incorporated by reference, was followed. Simultaneously with the RT-PCR, the amino terminal nucleotide sequence was altered to optimize protein expression in the vector used. See Garboczi et al., Proc. Natl. Acad. Sci. USA 89: 3429 (1992) incorporated by reference. Once this was done, the extracellular coding portion of the molecule was amplified, again using specific primers. The resulting construct was recloned into a vector which would produce a BirA biotinylation recognition site in frame at the 3'-end of the HLA-A*0201 heavy chain. The modified HLA-A*0201 and ß2 microglobulin were overexpressed in separate E. coli cultures. The resulting inclusion bodies were purified and the HLA and ß2 microglobulin recombinant proteins were solubilized into urea, and then refolded, in a refolding solution, at 4°C to form complexes. (The refolding solution contained 100 mM Tris, at pH 8.0, L-arginine, 400 mM, EDTA, 2 mM, reduced glutathione, 5 mM, oxidized glutathione, 0.5 mM, PMSF, 0.1 mM, HLA heavy chain, and β2 microglobulin 1 µM, and 10 µM of the peptide of interest). The refolding solution was concentrated to 7.5 ml, using standard techniques. Then, refolding buffer was exchanged with BirA reaction buffer (Tris 100 mM, pH 7.5, NaCl 200 mM, Mg Cl$_2$ 5mM, PMSF 100 µM, leupeptin 1 µM, and pepstatin 1 µM), the last three being added immediately before use.

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The complexes were then biotinylated with biotin holoenzyme synthase (the BirA

enzyme) by combining the refold mix containing the HLA-A2 complex with 50 µM enzyme,

100 mM biotin in 200 mM Tris, and 100 mM adenosine triphosphate. The mixture was

incubated overnight at room temperature. The biotinylated complexes were then purified, and

5   combined with phycoerythrin-labelled streptavidin, to produce tetrameric structures. These were

isolated, and reconstituted in small volumes, at a concentration of 1 mg/ml.

Example 2

Following isolation and reconstitution of the tetramers, these were tested to determine

if they could identify CTLs which express the cognate T cell receptor. To test this, monoclonal

10   CTL populations specific for SEQ ID NOS: 2, 4 and 5 (see infra), were used. In these

experiments, tetramers were prepared, using SEQ ID NOS: 3, 4 and 5 (see infra, but not SEQ

ID NO: 2) in a two color fluorescence assay with anti-CD8 antibodies, labelled with FITC. The

tetramers uniformly stained the CTLs, providing a signal two orders above background. The

specificity of CTL clone staining correlated well with the specificity of lytic activity displayed

15   by the same clones against ^{51}Cr labelled T2 cells sensitized with SEQ ID NO: 2, 3, or 4, infra.

Example 3

The peptides, which were used in examples 1 and 2, i.e.:

Glu Ala Ala Gly Ile Gly Ile Leu Thr Val   (SEQ ID NO: 2)

Glu Leu Ala Gly Ile Gly Ile Leu Thr Val   (SEQ ID NO: 3)

20   Tyr Met Asp Gly Thr Met Ser Gln Val   (SEQ ID NO: 4) and

Gly Ile Leu Gly Phe Val Phe Thr Leu   (SEQ ID NO: 5)

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are all known to bind to HLA-A2 molecules, and stimulate CTLs. SEQ ID NO: 2 is derived

from Melan-A; SEQ ID NO: 3 is an analogue of SEQ ID NO: 2; SEQ ID NO: 4 is derived from

tyrosinase 2 (Skipper et al., J. Exp. Med. 183: 527 (1996)); SEQ ID NO: 5 is a control peptide

from influenza matrix protein (Gotch et al., 1987, Nature 326; 881). Hence, they were used in

5   the formation of the tetramers discussed supra. The tetramers were then used to detect specific

T lymphocytes in tumor infiltrated lymph nodes ("TILNS" hereafter).

TILNS were surgically removed from ten melanoma patients, who had been typed as

HLA-A'0201 positive. The TILNS were treated in the same manner as the TILNS in Romero

et al., J. Immunol. 159: 2366-2374 (1997), incorporated by reference. Control lymph nodes from

10   normal lymph nodes ("NLNs" hereafter) were also prepared. The TILNs were treated to form

single cell suspensions, and were either not cultured, or cultured for anywhere from 1-22 days

for expansion of specific T lymphocytes in medium supplemented with rIL-2 and rIL-7, and then

samples containing from $0.5\text{-}1.0 \times 10^6$ were stained with fluorescent, anti-CD3, anti-CD8

antibodies, as well as the tetramers described supra.

15   The anti-CD3 antibody was labelled with PerCP (peridinin chlorophyll protein). The

anti-CD8 antibody was labelled with FITC. A total of 1-2 ug of the tetramer being tested, in 20

ul volumes of PBS, 0.02 mM, 0.2% bovine serum albumin, 0.2% sodium azide, was used. It was

combined with the cells, for 30 minutes, at 4-8°C. The cells were washed once, with 3 ml of the

same buffer, and were then analyzed immediately via flow cytometry.

20   A surprising high number of lymphocytes (0.12 to 21% of CD3$^+$ and CD8$^+$ lymph node

cells) specific for complexes of SEQ ID NO: 2 or 3 were found, in 11 of the 12 TILN samples

tested. The binding lymphocytes were CD3$^+$ CD8$^+$ lymph node cells. There were also high

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numbers of TILN cells which bound to the tetramers containing SEQ ID NO: 4, ranging from 0.14 to 0.63%.

Example 4

5   The samples discussed supra were analyzed further, emphasis being placed on TILN samples which had been incubated overnight (5 samples) or stained immediately (2 samples). Of these, the levels of SEQ ID NO: 3 tetramer binding lymphocytes ranged from 0.12 to 1.29%. There were no SEQ ID NO: 4/HLA-A2 positive TILNs found in these samples which had been cultured overnight. This suggests that highest levels of positive lymphocytes are achieved after short term in vitro culture.

10   ## Example 5

In order to confirm the suggestion outlined supra, cell suspensions were prepared from a heavily tumor infiltrated lymph node and a normal lymph node ("NLN") from the same patient. The suspensions were cultured, as described supra, overnight, for 15 days, or for 21 days and analyzed for tetramer staining as described supra. Tetramer positive cells rose from 0.12 to

15   12.2% in the TILNs, but from 0.14 to 0.81% in the NLNs when the 21 day cultured lymph node cells were used. What was more drastic, however, was the absolute number of tetramer positive cells. Specifically, the apparent number of SEQ ID NO: 3/HLA-A2 tetramer positive CD3⁻ CD8⁺ cells rose from 210 to about 1.8×10^5 after 15 days, and 5.38×10^5 after 21 days, representing 861 fold and 2562 fold expansions. Minor but significant expansion was seen in the NLNs, but

20   at two orders of magnitude less.

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Example 6

Studies were carried out to determine relationships between those TILN lymphocytes which bind the tetramers, and CTL effectors capable of killing tumor cells expressing Melan A antigen and to obtain highly homogenous populations of such CTL effectors.

5   To do so, TILN cells from a patient were cultured, for 21 days, as described supra, using recombinant IL-2 and IL-7, and were stained with anti-CD3, labelled with peridinin chlorophyll protein, and anti-CD8, labelled with FITC. These were then sorted into Melan A/HLA-A2 tetramer positive, and negative cells, as described supra. Chromium release assays (Romero et al. 1997, J. Immunol. 159: 2366) were carried out which showed that only the Melan-A/HLA-A2

10   tetramer positive T lymphocytes were able to kill T2 target cells sensitized with the peptide of SEQ ID NO: 3.

Example 7

To further characterize the functional antigen specificity, TILNs which were sorted as in example 5 were expanded for two weeks via non-specific stimulation with mitogen PHA, and

15   allogeneic, irradiated feeder cells. The sorted, expanded TILNs in a ^{51}Cr release assay as described supra against non-melanoma HLA-A2 expressing T2 cells, which had been sensitized with one of seven peptides known to bind to HLA-A2 molecules, and to generate CTLs. The assay was carried out under the conditions in example 6, supra.

It was found that lymphocytes which were specified to the tetramers containing the

20   decapeptide of SEQ ID NO: 3 only recognized this peptide. In contrast, tetramer negative cells did not recognize any of the peptides.

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It was also observed that the Melan-A specific cells did in fact kill the autologous tumor cell line efficiently.

Example 8

5   Apart from the importance of being able to visualize antigen specific CTLs, as is shown supra, it is important to determine if these CTLs have encountered the antigen in vivo. Prior work has established that expression of several antigens, including CD25, CD69, and CD45RO are associated with T cell activation in $CD8^+$ lymphocytes. These first two markers are expressed transiently, while CD45RO is expressed in a more stable manner. Hence, the staining method described supra was carried out, using anti-CD8 antibodies labelled with peridin

10   chlorophyll protein, anti-CD45RO, labelled with FITC, and the tetramers described supra. Ex vivo cells suspensions were prepared from two patients, and then studied, with a tumor infiltrated lymph node being tested with an unfiltrated node used for comparison.

It was found that the tetramer positive lymphocytes were predominantly, if not exclusively in the $CD45RO^+$, $CD8^+$ lymphocytes. This indicated that such lymphocytes had

15   encountered antigen in vivo and were therefore being activated at the tumor site.

Example 9

In view of the experiments described supra, additional work was carried out to determine if the tetramers could be used to isolated complex specific CTLs from circulating lymphocytes. To do this, cryopreserved peripheral blood lymphocytes were thawed (37°C, 3 minutes), then

20   washed twice with standard culture medium. Magnetic cell sorting was carried out to select $CD8^+$ cells, following standard techniques. The cells were then analyzed, using $CD45RA^-$

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specific antibodies labelled with cytochrome, and CD28 labelled with FITC, following the

protocols of example 8. A total of 0.11% of the CD8$^+$ PBLs were tetramer positive and, most

notably, most were CD28$^+$. Approximately 44% of the CD8$^+$, tetramer specific cells, were

CD45RA$^+$. This shows that uncultured, unstimulated blood lymphocytes can be assayed for

5   appropriate CTLs specific for the complex of interest.

Example 10

Further analysis were carried out with the tetramers of SEQ ID NO: 4. These stained

CTLs known to be specific for complexes of SEQ ID NO: 4 and HLA-A2, but not others. The

tetramer was then used to study PBMCs of individuals who were not known to be suffering from

10   influenza infection, to determine if the tetramers could be used to assay for low frequency CTLs,

like memory cells, ex vivo. The assay was carried out, as described in examples 8 and 9, supra.

The percentage of complex specific CD8$^+$ cells was considerably higher than the

percentage estimated previously using limiting dilution analysis, but are similar to the percentage

observed, using ELISPOT analysis, as per Lalvani et al., J. Exp. Med. 186: 859-865 (1997),

15   which is incorporated by reference.

Example 11

Studies were then carried out to phenotype the complex specific, CD8$^+$ cells. A large

number of PBMCs were obtained from an individual and, using the assay of example 10, supra,

the frequency of CD8$^+$ cells specific for the complexes was consistently found to be about

20   1/3700 of the PBMCs, and 1/2500 of small lymphocytes which are activated, or "memory" CD8$^-$

cells. When tested for cell surface markers, these were found to be CD45RO$^+$/CD45RA$^-$,

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consistent with such cells having previously encountered antigen. At least 74% of the positive

CTLs expressed the Vβ17 chain, as determined by cell surface staining, as compared to 4% of

the negatives, which confirms results shown by Lehner et al., J. Exp. Med. 181: 79-91 (1995).

Only 36% of the positive cells were CD28+, compared to 75% of the negative CTLs. Again, this

is consistent with the majority of the cells having reduced proliferative potential due to longer

replicative history. This shows that, using the triple staining methodology, a very pure cell

population can be obtained, by applying, e.g., FACS on peripheral blood lymphocytes, thereby

identifying CTLs.

Example 12

Specificity and activation of the CD8+ PBMCs discussed supra was studied using the

ELISpot technique, referred to supra. This technique yields spots of color whenever a CTL

releases gamma interferon. In control experiments, a CTL clone specific for SEQ ID NO:

4/HLA-A2 complexes was stained with the tetramer and anti-CD8. Then, 100 double positive

cells were sorted directly into duplicate wells of ELISPOT plates containing T2 cells, which had

been prepared with either SEQ ID NO: 5, or as a control, SEQ ID NO: 2. No gamma interferon

was released from controls, indicating that tetramer staining did not per se activate the cells. The

SEQ ID NO: 5 containing matrices, however, provoked gamma interferon release corresponding

to about one spot per CTL per day, indicating all cells had been activated by their cognate

peptide.

Following this, the PBMCs were stained, sorted into sets of 15 cells and duplicate

ELISPOT wells for 1 or 2 days of incubation with peptide pulsed targets. Interferon release was

observed only when the peptide of SEQ ID NO: 5 was used. Pulsing with other peptides did not

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result in gamma interferon release, nor did tetramer negative CD8⁺ cells release IFN-gamma on exposure to any peptide.

Since the number of spots formed was close to the number of PBMCs in the wells, this suggests that the majority of the tetramer positive, CD8⁺ cells were capable of responding within one day, thus supporting the concept that most circulating memory CD8⁺ cells are capable of rapid effector function when triggered by exposure to cognate peptide in the absence of cytokine, even when the presenting cells are not professional antigen presenting cells.

Example 13

Studies were then carried out to confirm the specificity of tetramer specific, CD8⁻ specific PBMCs, and to study proliferative potential. To do this, single PBMCs were sorted directly into cloning wells. After two weeks, 15 of 60 wells seeded with these cells contained proliferating blasts. The four most proliferative clones were tested in duplicate ELISPOT assays for specificity, along with one proliferating CD8⁻, tetramer negative clone. What was found was that all four of the CD8⁺, tetramer positive clones synthesized gamma interferon when exposed to targets pulsed with SEQ ID NO: 4, but not when pulsed with a control peptide. The tetramer negative, CD8⁺ cells failed to respond at all. Further, one of the clones was tested in a chromium release assay, where it specifically lysed target cells pulsed with SEQ ID NO: 5.

Example 14

The potential of tetramer based, FACS sorting in analyzing tumor specific CTLs responses was studied using tetramers containing SEQ ID NO: 2, as described supra. The tetramer was then used to stain and sort a polyclonal CTL line, which had been generated from

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an HLA-A2$^+$, melanoma infiltrated lymph node. Only a small percentage of cells (6%), could be double stained with the specific tetramers and antibody to CD8. The polyclonal line did kill A2 matched targets pulsed with SEQ ID NO: 2, although it was poor at killing A2 matched melanoma lines expressing Melan-A, and demonstrated small background killing of target cells

5   pulsed with a negative control.

An aliquot of the polyclonal line was then enriched for tetramer specific, CD8$^+$ cells by FACS sorting. This line killed melanoma lines and target pulsed with SEQ ID NO: 2 much more efficiently than the original polyclonal line, and showed no background killing. The specific cytotoxicity correlated entirely with the percentage which stained tetramers, indicating that

10   tetramer staining, followed by FACS sorting, can be used to generate highly effective, tumoricidal, CTL lines.

Example 15

These experiments describe in vitro expansion of Melan A specific precursors from PBMCs of melanoma patients.

15   Samples of PBMCs were taken from seven melanoma patients, using standard techniques, and were enriched for CD8$^+$ lymphocytes via magnetic cell sorting, to give populations of cells which were over 99% CD3$^+$/CD8$^+$.

These cells were then cultured, overnight, under standard conditions, and were then stained with tetramers of HLA-A* 0201 and SEQ ID NO: 3, which were labelled with

20   phycoerythrin, and anti-CD8 monoclonal antibodies (Staining was accomplished by combining the cells with 20μl of PBS with 2% FCS, and incubating for 15 minutes at 4°C, after which 20μl of anti-CD8 monoclonal antibodies labelled with FITC were added, and incubated for 30

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minutes at 4°C). Cells were then washed, once, with the buffer described supra, and analyzed

by standard flow cytometry.

The frequency of tetramer positive cells ranged from 0.03% to 0.09% of the total CD8⁺

population. These frequencies were close to the detection limit of the flow cytometer, which led

5   to the next experiments.

Example 16

In view of the low levels of positive cells found, PBMCs were taken from 3 of the 7

melanoma patients described supra, and were stimulated three times, at weekly intervals, with

either SEQ ID NO: 2 or SEQ ID NO: 3 pulsed, autologous PBMC cells. To elaborate, the

10   samples were stimulated, initially, by adding 1μM of peptide directly into the medium. Cultures

were then stimulated weekly by adding autologous PBMCs (3x10⁶ cells/well), which had been

pulsed for 2 hours at 37°C, in serum free medium, with 1μM of the peptide of SEQ ID NO:2 or

SEQ ID NO: 3 and 3μg/ml of human β2 microglobulin. These peptide pulsed cells were washed

extensively, irradiated (3000 rad), and adjusted to an appropriate volume before being added to

15   the responder cell population. Both IL-2 (10U/ml), and IL-7 (10ng/ml), were added during the

first two stimulation cycles, and IL-2 alone was added thereafter, at 100 U/ml.

The assay described in example 15 was carried out on the samples 7 days after each

stimulation cycle.

During the first week of stimulation, the total number of tetramer positive cells declined

20   consistently, increased moderately after the second stimulation, and significantly after the third

one. Stimulation with the peptide of SEQ ID NO: 3 resulted in more vigorous expansion than

did stimulation with SEQ ID NO: 2. The difference in stimulatory capacity was apparent after

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the first cycle and became very marked after the third one. The results are summarized in the following table.

PERCENT POSITIVE FOR TETRAMER STAINING AND CD8 ANTIBODY			
	WEEK 1	WEEK 2	WEEK 3
SEQ ID NO: 2	.30	.38	.69
SEQ ID NO: 2	.20	.35	2.1
SEQ ID NO: 2	.26	.34	.78
SEQ ID NO: 3	.47	2.3	2.8
SEQ ID NO: 3	1.6	5.4	29
SEQ ID NO: 3	.99	4.7	44

Example 17

Cultures were tested seven days after the third stimulation cycle for their capacity to lyse T2 cells, in the absence or presence of each of the two peptides. (T2 cells are known as cells which are HLA-A* 0201 positive, are defective in antigen processing ability, but present exogenously supplied peptides effectively). The assays were carried out by labelling the T2 cells with ^{51}Cr for 1 hour at 37°C, followed by two washes. Labelled target cells (1000 cells in 50μl) were then added to varying amounts of effector cells (in 50μl) in V-bottom microwells, either in the presence of 1μg/ml of the peptide of SEQ ID NO: 2, 1μg/ml of the peptide of SEQ ID NO: 3, or no peptide.

The effector cells had been preincubated for at least 20 minutes at 37°C in the presence of unlabelled K562 cells (50,000/well) to eliminate non-specific lysis due to NK-like effectors in the stimulated T cell populations. Chromium release was measured after 4 hours.

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The results are presented in figure 1. Frequency of HLA-A*0201/SEQ ID NO: 3 tetramer positive cells detected in the cultures correlated directly with peptide specific cytotoxicity. Also, CTLs which were generated following stimulation with SEQ ID NO: 3 cross recognized SEQ ID NO: 2.

5   **Example 18**

Additional experiments were carried out to document the specificity of CD8^{+}, HLA-A* 0201/peptide tetramer positive lymphocytes which had been generated supra.

In these experiments, PBMCs from one of the three patients described supra were stimulated with autologous PBMCs that had been pulsed with SEQ ID NO: 1, SEQ ID NO: 2,

10   SEQ ID NO: 3, the peptide Ala Ala Ala Gly Ile Gly Ile Leu Thr Val (SEQ ID NO: 37), Ala Leu Ala Gly Ile Gly Ile Leu Thr Val (SEQ ID NO: 38), or an immunodominant HLA-A2 restricted epitope from influenza matrix protein, presented as SEQ ID NO: 5, supra. (SEQ ID NOS: 37 and 38 are Melan A analogues, and hence are related to SEQ ID NOS: 1,2 and 3). The stimulation protocol was identical to that given in example 17, supra.

15   A small number of HLA-A* 0201/ peptide tetramer positive cells were detected at the end of the first stimulation cycle in the cultures stimulated using the influenza peptide, and was equivalent to the number of such cells detected in uncultured cells. The number declined after a second round of stimulation.

Expansion of the tetramer positive lymphocytes occurred with both SEQ ID NOS: 1 and

20   2, directly demonstrating the ability of the peptides to stimulate in vitro expansion of Melan-A specific lymphocytes in peripheral blood. Their stimulatory capacity appears to be similar.

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Notwithstanding this point, the increase in proportion of tetramer positive lymphocytes was much more pronounced when analogues of SEQ ID NO: 1 were used. The largest expansion occurred with SEQ ID NO: 38. The following table summarizes these results. The values show the percentage of cells which were positive both for the tetramer discussed supra, and the FITC

5   labelled, anti CD8 antibody.

Peptide

Tetramer Positive %	SEQ ID NO: 5	SEQ ID NO: 1	SEQ ID NO: 2	SEQ ID NO: 38	SEQ ID NO: 3	SEQ ID NO: 39
Week 1	.14	.23	.52	.69	.99	.99
Week 2	.09	.15	.25	1.1	1.2	2.4
Week 3	.01	1.4	3.2	16.0	9.0	31.0

Example 19

Following the work in example 18, the viable cells recovered after the third stimulation were titrated as effectors in a ^{51}Cr release assay of the type described supra.

15   Frequency of tetramer positive cells correlated directly with the level of specific cytotoxicity, and CTLs resulting from the analogue driven expansion effectively lysed target cells sensitized with SEQ ID NO: 2.

Example 20

Examples 15-19 show that it is possible to directly visualize antigen specific T

20   lymphocytes using flourescent tetramers, and to separate them from bulk cultures. They suggest

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the possibility of early stage, in vitro stimulation with peptide, and continued expansion in antigen independent fashion. This was tested in the experiments which follow.

Samples of CD8⁺, tetramer positive cells were isolated from each of the cultures described in example 18, seven days after the second cycle of stimulation. These sorted cells

5   were then expanded in vitro by stimulation with phytohemagglutinin, and the resulting cells were tested for their lytic activity on T2 cells, in the same manner described supra, in the absence or presence of the peptide used in the particular culture from which the cells were taken originally, or SEQ ID NO: 2. Concurrently, the ability of these CD8⁺ cells to lyse tumor cells was tested, using Melan-A positive tumor cell line Me 290 as the target.

10   The results, presented in figure 2, show that all the cell populations exhibited a high degree of specific lysis against target cells which had been pulsed with the corresponding stimulatory peptide and with cells pulsed with SEQ ID NO: 2. They also exhibited high tumoricidal activity. Me 290 is illustrative of additional Melan-A positive tumor cell lines which were lysed. Melan-A negative tumor cell lines were not lysed. Effector:target ratios necessary

15   for achieving half maximal lysis ranged from 3:1 to 15:1.

Example 21

The relative avidity of peptide antigen recognition of the different cell populations which had been obtained was assessed in CTL assays, following standard protocols. Specifically, 1000 target cells (50μls) were incubated with varying concentrations of peptides (50μl) for 15 minutes

20   at room temperature before effector cells were added. The concentration of each peptide required to achieve 50% maximal lysis of target was determined, using standard methods. To make comparison easier, relative activity of peptide was calculated as the 50% concentration of

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SEQ ID NO: 1, divided by the 50% concentration of the tested peptide. Figure 3 shows these results. These results are also presented in the table which follows. The values are the peptide concentrations required for 50% maximal activity.

The relative avidity of the different lines for SEQ ID NO: 1 and SEQ ID NO: 2 were remarkably similar, except for the line obtained using SEQ ID NO: 39, which recognized SEQ ID NO: 1 about 3 fold less efficiently, and SEQ ID NO: 2 5-7 fold less efficiently as compared to other lines.

The results are also presented in terms of relative antigenic activity. Note that, regardless of the peptide used for in vitro expansion, all lines recognized SEQ ID NO: 2 more efficiently than SEQ ID NO: 1. Analogues were recognized more efficiently than both parental sequences by all lines, notwithstanding some differences in relative antigenicity.

Preference of a line for a certain analogue did not correlate with the analogue used to generate the line: in all cases, SEQ ID NO: 38 was the peptide most efficiently recognized.

Culture stimulated with:

	Melan-A 27-35 (SEQ ID NO: 1)	Melan-A 26-35 (SEQ ID NO: 2)	E26A (SEQ ID NO: 37)	A27L (SEQ ID NO: 3)	E26A/A27 L (SEQ ID NO: 38)
Peptide [nM] 50%:					
Melan-A 27-35 (SEQ ID NO: 1)	27	22	18	37	65
Melan-A 26-35 (SEQ ID NO: 2)	3	3	5	5	20
E26A (SEQ ID NO: 37)	0.09	0.14	0.12	0.19	1

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	Melan-A 27-35	Melan-A 26-35	E26A	A27L	E26A/A27L
A27L (SEQ ID NO: 3)	0.15	0.2	0.2	0.16	0.4
E26A/A27L (SEQ ID NO: 38)	0.02	0.01	0.008	0.005	0.5

Relative antigenic activity:

	Melan-A 27-35	Melan-A 26-35	E26A	A27L	E26A/A27L
Melan-A 27-35 (SEQ ID NO: 1)	1	1	1	1	1
Melan-A 26-35 (SEQ ID NO: 2)	9	7	4	7	3
E26A (SEQ ID NO: 37)	300	157	183	194	65
A27L (SEQ ID NO: 3)	180	110	110	231	162
E26A/A27L (SEQ ID NO: 38)	1350	2200	2750	7400	130

Example 22

The peptide presented supra as SEQ ID NO: 4 is derived from the tyrosinase molecule, which is known to function as a tumor rejection antigen precursor, with the peptide of SEQ ID NO: 4 functioning as a tumor rejection antigen. See, e.g., Brichard, et al., J. Exp. Med. 178:489 (1993); Wölfel, et al., Eur. J. Immunol. 24:759 (1994). Work by others has focused on study of the binding properties of an analog of this peptide, i.e., one where asparagine at position 3 of the peptide, has been replaced by aspartic acid. (See SEQ ID NO: 22, supra). This modification occurs naturally, via post translational processes. Skipper, et al., J. Exp. Med. 183:527 (1996) have shown that this difference significantly affects T cell recognition. The experiments which follow are directed to a determination of what role single amino acid side chains contribute to the interaction between SEQ ID NO: 4 and HLA-A* 0201 molecules.

First, single alanine substituted derivatives of SEQ ID NO: 4 were prepared. i.e.:

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Ala Met Asp Gly Thr Met Ser Gln Val (SEQ ID NO: 39)

Tyr Ala Asp Gly Thr Met Ser Gln Val (SEQ ID NO: 40)

Tyr Met Ala Gly Thr Met Ser Gln Val (SEQ ID NO: 41)

Tyr Met Asp Ala Thr Met Ser Gln Val (SEQ ID NO: 42)

5   Tyr Met Asp Gly Ala Met Ser Gln Val (SEQ ID NO: 43)

Tyr Met Asp Gly Thr Ala Ser Gln Val (SEQ ID NO: 44)

Tyr Met Asp Gly Thr Met Ala Gln Val (SEQ ID NO: 45)

Tyr Met Asp Gly Thr Met Ser Ala Val (SEQ ID NO: 46)

Tyr Met Asp Gly Thr Met Ser Gln Ala (SEQ ID NO: 47)

10   These peptides were synthesized using standard methodologies, and were then tested in

a functional competition assay. To elaborate, the assumption was that these peptides would bind

to HLA-A*0201 molecules. It is known that SEQ ID NO: 1 binds to HLA-A*0201. Hence, T2

cells, described supra, were labelled with ^{51} Cr in the presence of a well known monoclonal

antibody, i.e., W6/32, which binds MHC - Class I molecules.

15   Then, varying concentrations of test peptides SEQ ID NOS: 39-47 were added, in 50 μl

aliquots, to the labelled T2 cells (1000 cells/well), for 15 minutes at room temperature, after

which a suboptimal dose of SEQ ID NO: 1 (125nM in 50 μl), was added, together with 5000

cells/well of an HLA-A*0201/SEQ ID NO: 1 restricted CTL clone, as taught by Valmori, et al.,

J. Immunol 160: 1750 (1998). Chromium release was measured after 4 hours of incubation at

20   37°C. The concentration of each competitor peptide required to achieve 50% inhibitions of target

cell lysis was determined. This is indicated in the Table which follows, as [nM] 50%. The

comparison is facilitated by presenting relative competitor activity as [nM] 50% of SEQ ID NO:

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4. This peptide's activity is assigned arbitrary competitor activity of 1. As a control, an influenza matrix peptide was used.

It was found that SEQ ID NO: 4 displays intermediate competitor activity, i.e., to secure 50% of maximal lysis, a concentration of 100nM was needed, as compared to 6nM for SEQ ID NO: 5, known to be a high affinity binder. It was surprising that changes at putative anchor positions 2 and 9 had marginal impact. Changes at positions 7 and 8, for example, had a much greater impact.

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LIID 5545.1-JEL/NDH

Table

Binding of single A substituted Tyrosinase 368-376 peptide variants to HLA-A* 0201

Peptide	Sequence	Competitor activity [nM] 50%	Relative competitor activity
Tyrosinase 368-376	YMDGTMSQV (SEQ ID NO: 4)	102	1
analogues	AMDGTMSQV (SEQ ID NO: 39)	200	0.5
	YADGTMSQV (SEQ ID NO: 40)	300	0.3
	YMAGTMSQV (SEQ ID NO: 41)	90	1.1
	YMDATMSQV (SEQ ID NO: 42)	100	1.0
	YMDGAMSQV (SEQ ID NO: 43)	80	1.3
	YMDGTASQV (SEQ ID NO: 44)	200	0.6
	YMDGTMAQV (SEQ ID NO: 45)	50	2.0
	YMDGTMSAV (SEQ ID NO: 46)	30	3.3
	YMDGTMSQA (SEQ ID NO: 47)	80	1.3
Influenza A matrix 58-66	GILGFVFTL (SEQ ID NO: 5)	6	17

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Example 23

It had been observed previously that complexes formed between HLA-A*0201 molecules and SEQ ID NO: 2 are unstable, dissociating completely in under one hour when incubated at 37°C. Stability studies were carried out on complexes of HLA-A*0201 and SEQ ID NO: 4. In brief, T2 cells were loaded with saturating concentrations (10μM) of one of SEQ ID NO: 4, SEQ ID NO: 2, or control peptide SEQ ID NO: 5. Following overnight incubation with the peptides and β2-micro-globulin (3μg/ml) in serum free medium, the T2 cells were treated with ementine (10⁻⁴ M) to inhibit protein synthesis, and incubated at 37°C. The cells were stained, at different time periods, by incubating an aliquot with anti-HLA-A2 specific monoclonal antibody BB7.2, to measure HLA-A2 antigen expression. The results were expressed as relative complex stability, defined by the formula:

$$100\% \times \frac{[\text{Mean fluorescence test peptide - background mean fluorescence}]}{[\text{Mean fluorescence SEQ ID NO: 48 - background mean fluorescence}]}$$

"Background" refers to fluorescence values obtained on T2 cells treated similarly but for absence of exogenous peptide.

Figure 4 presents these results. They confirm the instability of HLA-A2/SEQ ID NO: 1 complexes, and show that the complexes of HLA-A2 and SEQ ID NO: 4 are stable over a 6 hour period.

Example 24

It has been observed, by Romero, et al., J. Exp. Med. 188:1641 (1998) incorporated by reference, and in the preceding examples, that significant numbers of HLA-A2/SEQ ID NO: 4

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positive, $CD8^+$ T lymphocytes can be detected in short term cultured TILNs from HLA-A*0201

positive melanoma patients. Studies were carried out to assess the antigenic and functional

specificity of such positive lymphocytes.

In brief, positive and negative fractions were separated via flow cytometry sorting, using

5   tetramers of HLA-A2/SEQ ID NO: 4, and CD8 specific monoclonal antibodies, as described

<u>supra</u>. Following separation, cells were expanded by stimulation with PHA and irradiated

allogeneic PBMCs, as described <u>supra</u>, over a two week period. The fractions were tested for

their ability to lyse ^{51}Cr labelled T2 cells in the absence or presence of $4\mu M$ of SEQ ID NO: 4.

The $CD8^-$, tetramer positive fraction lysed the T2 cells in the presence of SEQ ID NO: 4, but not

10   its absence. Negative fractions did not lyse the target.

Example 25

These experiments were designed to obtain monoclonal T cell populations specific for

SEQ ID NO: 4 containing complexes. To do this, limiting dilution cultures were set up

immediately after the cell sorting described in example 24. Limiting dilution was carried out

15   following Valmori, et al., J. Immunol. 160:1750 (1998), incorporated by reference, using

irradiated allogeneic PBMCs, EBV transformed B lymphocytes, PHA and recombinant IL-2.

Six specific CTL clones were secured, and a seventh clone was obtained from

unfractionated TILNs after in vitro stimulation in an independent cloning experiment. These

constitute monoclonal T cell populations.

20   ### Example 26

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These experiments were designed to analyze both polyclonal, monospecific T cell populations, and the monoclonal T cell populations described supra, for tumor antigen recognition, avidity, and fine specificity. This example describes the antigen recognition experiments.

5   Three cell lines were used. NA8-MEL is an HLA-A*0201 positive melanoma cell line which does not express tyrosinase. NA8-MEL tyr⁺ is a cell line derived by transfecting NA8 with cDNA encoding tyrosinase. Cell line Me 290 described supra as an HLA-A* 0201 positive, Melan-A⁺ cell line is also tyrosinase⁺.

Samples of each of these lines were combined with one of a polyclonal, monospecific

10   CTL population obtained by using the tetramers of SEQ ID NO: 4 and HLA-A* 0201, supra, or the monoclonal T cell populations described supra.

The melanoma cell lines and T cells were combined, at varying ratios, either with or without the peptide, at 1μM concentration. The tumor cells had been labelled with ⁵¹Cr prior to combining them with the T cells. ⁵¹Cr release was measured after 4 hours of incubation, using

15   the same parameters as are described supra.

These results are set out in figure 5. The panel labelled Tyr. tetr⁺ shows data from the polyclonal cell population. The others are the monoclonal lines described supra.

All of the clones and the polyclonal population lysed the NA8-MEL cell line, which is tyrosinase negative, only when the peptide of SEQ ID NO: 4 was added. The other lines, i.e.,

20   Me 290 and NA8-MELtyr+ were lysed equally well, both in the presence of the peptide and without it.

Example 27

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Fine antigenic specificity of the T cell populations discussed in example 26 was analyzed, by quantitating the relative antigenic activity of the variants set out in SEQ ID NOS: 39-47 in standard CTL assays. These were carried out by using a ^{51}Cr release assay, as described supra. Relative avidity was determined by comparison to the concentration of the peptide of SEQ ID

5   NO: 4 required to obtain half maximal lysis at an effector:target ratio of 10:1. The results are presented in the following Table.

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LJD 5545.1-JEL/NDH

SEQ ID NO	Peptide	A2/Tyr	CTL clone:						
	Tyrosinase 368-376 [nM] 50%	Tetr' population	1A12/1	1B3/1	1D5/1	1E12/0.	1F11/	1G4/1	LAU 132/2
4		4	70	4	6	3 35	1 200	20	15
	Peptide sequence					**Relative antigenic activity**			
4	YMDGTMSQV	1	1	1	1	1	1	1	1
39	AMDGTMSQV	0.05	<0.003	0.08	0.01	<0.003	1	0.02	<0.015
40	YADGTMSQV	1	1	0.2	1	0.1	5	0.5	2.1
41	YMAGTMSQV	0.006	<0.003	<0.0004	0.06	0.08	<0.02	<0.00 2	0.25
42	YMDATMSQV	8	<0.003	10	0.0002	<0.003	100	5	<0.015
43	YMDGAMSQV	0.08	0.07	<0.0004	0.2	<0.003	<0.02	<0.00 2	3.7
44	YMDGTASQV	<0.0004	<0.003	<0.0004	0.02	5	<0.02	<0.00 2	<0.015

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45	YMDGTMAQV	<0.0004	<0.003	<0.0004	0.04	<0.003	<0.02	<0.002	<0.015
46	YMDGTMSAV	0.05	<0.003	<0.0004	1	0.05	<0.02	<0.002	100
47	YMDGTMSQA	0.06	<0.003	0.05	1	0.5	0.5	0.2	0.03

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Each clone displayed unique fine specificity. Replacement of residues at positions 2 and 9 did not drastically affect antigen recognition in the majority of cases, which is consistent with lack of direct action between side chains located at these positions, and T cell receptors, as suggested by Madden, et al., Cell 75:693 (1993). The results indicate that position 7 of the peptide (a Ser residue), was important, while replacement at different positions gave highly variable results. The net result is a showing of wide heterogeneity in the recognition of epitopes by CTLs specific to SEQ ID NO: 4, and derived from a single, infiltrated lymph node.

Example 28

Example 15, supra, showed how tetramers of HLA-A* 0201 and a Melan-A derived peptide could be used to measure the frequency of CD8⁺ T lymphocytes in a population. Similar experiments were carried out using tetramers of HLA-A* 0201 and SEQ ID NO: 4.

The same protocol as was used in example 15, but for a different tetramer, was carried out on highly enriched CD8⁺ T cells from PBMCs of 10 HLA-A* 0201 positive melanoma patients. Seven samples showed frequencies of tetramer⁺ cells ranging from less than 0.01% and up to 0.03%. These ranges were not very much above the level of detection afforded by the flow cytometry assay, so the enriched, CD8⁺ T lymphocytes for all 10 samples were stimulated with SEQ ID NO: 4, using the protocol of example 16, supra, with minor modifications. Specifically, cells were initially stimulated with 100 μM of peptide directly into the culture medium, and weekly stimulations were carried out with additional 100μM samples of peptide. Additionally, IL-2 (100 U/ml) and IL-7 (10 ng/ml) were added during the first stimulation cycle, and IL-2 alone (100 U/ml) thereafter. Cultures were monitored seven days after stimulation for the presence of tetramer positive cells.

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The results are summarized in the following Table. What is interesting is that positive

lymphocytes were detectable in six out of 10 samples after only short term culture. (The other

four samples were positive, but at the limits of detection for the assay).

SAMPLE #	% POSITIVE Before Stimulation	% POSITIVE After Stimulation
1	0.01	0.01
2	0.00	0.03
3	0.01	0.17
4	0.03	0.27
5	0.01	0.21
6	0.02	2.20
7	0.00	0.01
8	0.00	0.03
9	0.01	0.39
10	0.01	0.40

The values refer to the percentage of A2/SEQ ID NO:4 tetramer positive T cells, relative to the

total number of CD8$^+$ lymphocytes.

Example 29

In additional experiments, tetramers of HLA-A *0201 molecules and SEQ ID NOS: 2,

4 and now SEQ ID NO: 9, i.e., MAGE-3 derived peptide FLWGPRALV, described supra, were

made, as described in the preceding examples. These tetramers were then used to stain CTLs,

as described in the previous examples. To review, cell samples were stained with PE labelled

tetramer for 15 minutes at 37°C, after which tricolor labelled, anti-CD8$^+$ antibodies were added,

on ice, for 15 minutes. Following this, the materials were washed extensively, and then analyzed

by FACS.

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The tetramers stained CTLs which had previously been identified as being specific for complexes of HLA-A *0201 and the peptide used, but did not stain with other, non-specific peptides.

Example 30

These experiments describe staining of different types of samples from different patients.

Five patients were typed for expression of tumor antigens, following standard methodologies. Different types of samples were taken from these patients, all of whom had histologically confirmed, malignant melanoma. The characteristics of the patients and samples involved are set forth in the following table:

patient	sex	age	stage	metastases	therapy	tumor antigens expressed	sample processed for cloning
L02	F	65	IV	skin, lung, adrenals	surgery dacarbazine	melan-A tyrosinase	peripheral blood (PBL)
MM14	F	44	III	lymph nodes	surgery	melan-A tyrosinase	tumour-infiltrated lymph node (LN)

patient	sex	age	stage	metastases	therapy	tumor antigens expressed	sample processed for cloning
MM15	F	59	IV	skin, lung	surgery dacarbazine BCNU, cisplatin, tamoxifen gamma-interferon	melan-A tyrosinase	skin metastasis
MM18	M	51	III	lymph nodes	surgery	melan-A	tumour infiltrated lymph node (LN)
DECH	F	39		lymph nodes	?	MAGE3	peripheral blood (PBL)

Patient L02 was leukapheresed, and PBLs from this patient were cryopreserved immediately. These PBLS were analyzed after thawing and overnight culture in Iscove's medium with 5% human serum added. Two additional PBL samples were taken over a period of 3 weeks for comparative analysis.

In the case of the patient referred to as DECH, PBLs were separated from a blood sample, and cryopreserved. Following thawing, these PBLs were pulsed with 10μM of SEQ ID NO. 9

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in "CTL medium" (Iscove's medium, 5% human serum, IL-2 at 100 U/ml), plus IL-7 (10ng/ml).

After this, they were cultured in CTL medium for 2 weeks until they were analyzed.

Patient MM14 and MM18 had melanoma - infiltrated lymph nodes removed from the left

or right axillae, respectively, disrupted, and cultured in the CTL medium described supra, plus

5   IL-7 (10ng/ml). Any proliferating blasts were expanded in the CTL medium for 14 days (for

MM14), or 23 days (for MM18), before analysis.

In the case of MM15, a skin metastasis was removed from the left shoulder, mechanically

disrupted, and cultured in the medium described supra for 13 weeks. There was a small

population of TILs, which was stimulated with PHA (5μg/ml), and expanded in medium for two

10   more weeks before analysis.

Tetramer staining was carried out as described supra, using appropriate tetramers. In

other words, tetramers containing SEQ ID NO. 9 were used in MAGE-3 positive samples from

DECH. Melan-A positive patient samples were screened with tetramers containing SEQ ID NO.

3, while tyrosinase positive patient staples were screened with tetramers containing SEQ ID NO.

15   4.

Tissue culture of tumor infiltrated lymph nodes of MM14 revealed CD8$^+$ cells specific

for both the tyrosinase and Melan-A peptides. The cultured LNs from MM18 were positive for

SEQ ID NO. 3 only. Patient L02 had readily detectable CD8$^+$ cells specific to SEQ ID NO: 3

in peripheral blood at a frequency similar to the frequency of influenza specific CTLs in normal

20   patients, as described by Dunbar, et al., Curr. Biol 8:413 (1998). This frequency remained fairly

constant over the 3 week period of analysis. Patient DECH showed no CD8$^+$ cells specific for

SEQ ID NO: 9 in PBLs on direct analysis; however, two weeks after a single pulse with the

peptide, a small population of cells were detected. Finally, patient MM15 showed very few

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CD8$^+$ cells in a PHA stimulated culture from skin metastasis; however, a high portion of these

were stained by the tetramers containing SEQ ID NO. 3.

Example 31

The preceding example described how CD8$^+$ cells were identified. In these experiments,

5   CTLs are cloned, directly, from these CD8$^+$ populations.

To do this, single cells were sorted, directly, into U bottom, 96 well plates coated

previously with anti-CD3 and anti-CD28 antibodies (both at 100 ng/ml), in PBS, containing 10^5

irradiated B cells in CTL medium, as described supra, plus IL-4 (20ng/ml). The anti-CD3

provides a solid phase mitogenic signal, and the anti-CD28 antibodies were provided in case the

10   cells were capable of costimulation through CD28.

The cloning plates were incubated at 37°C in 5% CO$_2$ for 10-14 days, without any

manipulation. Then, they were restimulated using PHA (5µg/ml), together with irradiated,

allogenic PBLs and B cells. All clones were expanded to at least 5x10^6 cells, in order to permit

characterization and banking. Several of the clones were stimulated further, in order to test

15   proliferative potential, and the total potential yield was calculated from the number of CTLs

generated in culture, and the number banked.

The table within follows summarizes these results. Essentially, between 2 and 13% of

sorted clones (average: 6.5%) expanded sufficiently to cryopreserve. Additionally, about three

times as many proliferated to a lesser degree.

20   The expanded clones were then tested for antigen specificity using either tetramers as

described supra, with confirmance by a ^{51}Cr release assay and ELISPOT analysis.

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With respect to the ^{51}Cr release assay, this was carried out by T2 cells which had been pulsed with relevant peptide (SEQ ID NOS: 3, 4 or 9), or a negative control, as well as cell lines SK-Mel-29 and SK-Mel 23, both of which are HLA-A2.1$^+$, and express both Melan-A and tyrosinase. The lysis percentage was calculated by the formula:

5

$$100 \times \frac{\text{(specific release - spontaneous release)}}{\text{(maximal release - spontaneous release)}}$$

Thirty three of thirty four of the clones were positive for their antigen, showing that the direct cloning was extremely efficient, as compared to methods where the vast majority of cloned cells do not have the desired specificity. Ten of these were tested by ^{51}Cr release, and three of these

10   were tested via ELISPOT. All showed specificity for relevant peptide.

With respect to cloning efficiency, no clear differences were seen, although peptide stimulated cultures required the greatest number of starting lymphocytes to generate clones:

sample	manipulation	tetramer	clones sorted	clones expanded (%)	antigen-specific expanded clones (%)	starting lymphocytes per specific clone
MM14 LN	IL-7, IL-2	A2/melan-A	60	8 (13%)	8 (100%)	25,000
MM14 LN	IL-7, IL-2	A2/tyrosinase	63	1 (2%)	1 (100%)	78,750
MM18 LN	IL-7, IL-2	A2/melan-A	175	11 (6%)	11 (100%)	53,030
L02 PBL	nil	A2/melan-A	60	7 (12%)	7 (100%)	14,290
DECH PBL	PEPTIDE, IL-2, IL-7	A2/MAGE3	110	2 (2%)	2 (100%)	183,330
MM15 metastasis	PHA, IL-2	A2/melan-A	80	7 (9%)	6 (86%)	1,875

Example 32

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The expanded CTLs were then tested for surface phenotytpes via FACS, using FITC

labelled antibodies against CD62L (L-Selectin), CD11b, CD44, CD45RO, CD45RA, TCRαβ,

and CD 49a-e., inclusive (integrin-B1-6, VLA 1-6), followed by goat-antimouse Ig. Also, anti-

CLA (cutaneous lymphocyte antigen), and FITC labelled mouse-antirat IgM labelled with FITC,

5   were used.

All clones tested were CD8^{+}, and TCRαβ^{+}, which was to be expected. They were also

all CD45RO^{-}, CD45RA^{-}, CD44^{+} and CD62L^{-}, which is consistent with previous antigenic

stimulation. The lack of CD62L on surfaces suggested that if these clones were infused during

immunotherapy, they would not home to lymph nodes via high endothelial venues, but would

10   traffic through peripheral circulation. The clones were CD11b and CD49d positive, which is

consistent with cells capable of migrating through activated vascular endothelium which express

VCAM-1 – a phenotype associated with metastatic melanoma (Rice, et al., Science 246:1303

(1989); Rice, et al., J. Exp. Med. 171:1369 (1990)).

A minority of clones were CLA positive suggesting that clones could be selected which

15   would or would not home to skin following infusion. See Picker, et al., Nature 349:796 (1991);

Ogg, et al., J. Exp. Med. 188:1203 (1998). Hence, it may be possible to pre-select clones for

immunotherapy according to their homing markers, via triple staining for surface molecules prior

to cloning. Also, clones with the best proliferative potential might be pre-selected by only

sorting tetramer [+] cells which express markers, such as CD28.

20   **Example 33**

In this example, and the examples which follow, additional characterization studies were

carried out on both melanoma patients and healthy volunteers.

43

Patients and donors were selected based upon HLA-A2 expression, determined by using

allele specific mAb BB7.2. PBMCs were isolated from samples, using standard methods. The

CD8+ cells in these samples were purified via two rounds of positive selection, using magnetic

beads, as described supra. These cells were greater than 98% CD3+CD8+.

5   These CD8+ cells were then stained with tetramers of HLA-A*0201 and SEQ ID NO: 3,

SEQ ID NO: 4 or control peptide SEQ ID NO: 5 and anti-CD8+ monoclonal antibodies labelled

with FITC and Cy-Chrome (a tandem flourescent conjugate system) (10^6 cells added to 50 μl of

PBS containing the antibodies and the tetramers, as well as 2% azide and 2% BSA. The

incubation period was 40 minutes, at a temperature of 4°C). Following the incubation, the cells

10   were washed, once, in the buffer used for the binding step, and then analyzed immediately via

FACS.

It was found that CD8+ cells specific to tetramers containing either SEQ ID NO: 3 or

control peptide SEQ ID NO: 5 were present in both melanoma patients and healthy subjects.

CD8+ cells specific to tetramers containing SEQ ID NO: 4 were not found. Note,

15   however, that these samples had not been stimulated with antigen in vitro. As was shown, supra,

even a short period of in vitro stimulation with antigen was sufficient to cause expansion of CD8+

cells specific to SEQ ID NO: 4 to the point where they could be detected in 6 of 10 patients

tested.

Example 34

20   A series of experiments were then carried out to determine levels of non-specific, HLA-

A2 tetramer positive CD8+ cells in healthy, HLA-A2 negative patients. The same tetramers as

are described in example 33, supra, were used, and the same protocol for separation and analysis

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were used. These results are set out in the table which follows. For comparison, tests were also

run on samples taken from HLA-A2 positive, healthy donors, HLA-A2 positive melanoma

patients, and HLA-A2 positive patients who suffered from both melanoma and vitiligo.

Percentage of circulating HLA-A2 Melan-A$_{26-35}$ A27L tetramer and Flu-MA$_{58-66}$ tetramer in CD8 T cells

5 HLA-A2 healthy donors			HLA-A2 healthy donors			HLA-A2⁺ melanoma patients			HLA-A2 melanoma patient + vitiligo		
Name	Melan-A	Flu-MA	Name	Melan-A	Flu-MA	Name	Melan-A	Flu-MA	Name	Melan-A	Flu-MA
HD 001	0.01	0.01	HD 006	0.05	0.11	LAU 50	0.03	0.41	LAU 155	0.12	0.07
HD 004	0.00	0.00	HD 007	0.01	0.07	LAU 56	0.08	0.13	LAU 156	0.40	0.18
HD 048	0.01	0.00	HD 008	0.06	0.02	LAU 97	0.07	0.10	LAU 269	0.16	0.02
HD 108	0.01	0.00	HD 099	0.08	0.07	LAU 132	0.07	0.05			
HD 143	0.03	0.00	HD 301	0.02	0.09	LAU 198	0.02	1.65			
HD 222	0.02	0.00	HD 304	0.03	0.05	LAU 203	0.07	0.03			
HD 299	0.01	0.00	HD 329	0.10	0.17	LAU 212	0.03	0.05			
HD 347	0.01	0.00	HD 421	0.11	0.03	LAU 233	0.21	0.08			
HD 408	0.01	0.00	HD 422	0.02	0.49	LAU 240	0.04	nd			
			HD 604	0.21	0.13	LAU 267	0.04	nd			
Mean	0.014	0.001	Mean	0.07	0.12	Mean	0.07	0.31	Mean	0.23	0.09
S.D.	0.008	0.003	S.D.	0.06	0.14	S.D.	0.06	0.55	S.D.	0.15	0.08
C.O.	0.036	0.011									

The lower detection limit for CD8⁺ cells which reacted with tetramers of SEQ ID NO:

3 was about 0.04%, while it was under 0.02% for CD8⁻ cells positive for SEQ ID NO: 5

containing tetramers. These limits of detection are lower than limits of detection for TILS, as

shown in Romero, et al., J. Exp. Med. 188: 1641-1650 (1998), due to differences in the type of

sample assayed. Of the 13 melanoma and melanoma/vitiligo patients tested, 10 showed

significant numbers of HLA-A2/SEQ ID NO: 3 positive CD8⁺ cells, while six of ten healthy

HLA-A2 positive donors showed significant numbers as well. Note that these CD8⁻ cells were

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almost all naive cells, as compared to the mix of naive and antigen experienced cells found in

melanoma patients.

Example 35

Prior work has established that the CD45RA and CD45RO isoforms can be used to

5   identify naive and memory T cells, respectively. See Young, et al., Eur. J. Immunol. 27: 2383-

2390 (1997), incorporated by reference. It has also been shown that circulating, CD28⁻ CD8⁺ T

cells present direct, ex vivo cytolytic activity (Azuma, et al., J. Immunol. 150: 1147-1159 (1993),

and Hamann, et al., J. Exp. Med. 186: 1407-1418 (1997), have proposed that such cells

correspond to effector type CTLs. In view of these prior observations, individuals who presented

10   significant amounts of circulating cells positive for tetramers of SEQ ID NO: 3 or SEQ ID NO:

5 were phenotyped for CD28, CD45RA, and CD45RO.

To do this, CD8⁺ lymphocyte populations were purified, as described _supra_. The highly

purified samples were divided into two, and then contacted with one of the tetramers described

supra, and with antibodies against CD45RA labelled with Cy-Chrome, and either antibody

15   against CD45RO labelled with FITC, or antibody against CD28 labelled with FITC. The

procedures for carrying out the staining are described, _supra_, and are not repeated here.

Virtually all circulating CD8⁺ T cells from healthy donors were positive for CD28, and

CD45RA, and negative for CD45RO when the cells were positive for tetramers of SEQ ID NO:

3. In contrast, CD8⁺ T cells which were positive for SEQ ID NO: 5 tetramers were CD28 and

20   CD45RO positive, and CD45RA negative (i.e., CD45RA^low). This suggests that the CD8⁺ T cells

specific for SEQ ID NO: 5 were antigen experienced memory cells, which is compatible with the

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idea that SEQ ID NO: 5 represents a recall antigen. The phenotype of the CD8+ T cells from healthy patients, which are specific to SEQ ID NO: 3, in contrast, suggests a naive phenotype.

This analysis was extended to melanoma patients. The majority of circulating CD8+ T cells specific for SEQ ID NO: 3 containing tetramers were CD28 and CD45RA positive, but

5   CD45RO negative. The results are presented graphically in figure 6.

Of the ten melanoma patients tested, the circulating CD8+ T cells specific for SEQ ID NO: 3 in seven presented a uniformly naive phenotype (i.e., CD28 and CD45RA positive, CD45RO negative), a phenotype identical to that of healthy donors' CD8+ T cells. Three of these melanoma patients either displayed greater than 35% memory cells (CD28 and CD45RO

10   positive, CD45RA negative), or more than 90% effector-like cells (CD28 negative, CD45RA intermediate levels, CD45RO negative).

Phenotype and frequency of CD8+ T cells positive for Melan-A tetramers were not correlated, in general. For example, the memory cells found in the two melanoma patients were not found at increased frequencies, while the high frequencies of CD8+ T cells positive for the

15   Melan-A containing tetramers were not of memory phenotype.

When the CD8+ T cells which were positive for tetramers containing SEQ ID NO: 5 were analyzed, the memory phenotype discussed _supra_ was found in 11 of the 13 melanoma and melanoma/vitiligo patients tested, as well as in all healthy donors. There was some heterogeneity in two of the melanoma patients.

20   **Example 36**

In these experiments, the staining methodology, limiting dilution assays, and the ELISPOT assay technique, all of which are described, _supra_, were compared.

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To start, sorted cells from two healthy donors (HD 329 and HD 604, in the table which

follows), and two melanoma patients (LAU 132 and LAU 203), were compared, using the

functional assays described supra.

In the two healthy patients and one of the melanoma patients, nearly all cells were

5   CD45RA⁻

(95, 95 and 94%), while only 54% of the second melanoma patient's cells were CD45RA⁺.

When samples were tested via limiting dilution, almost all of the Melan-A specific CTL

activity was found in the CD45RA⁺ naive subsets (98, 99 and 90%), while 73% of the CTL

activity for patient LAU 132 was found in the CD45RA⁻ (CD45RA^low) subset. Distribution of

10   CTL precursors among naive and memory subsets, as analyzed by LDA, paralleled the results

obtained using the flow cytometry assays; however, frequency of the CTL precursors was

underestimated by LDA, especially in the CD45RA⁺ population. The level of underestimation

was by a factor of 3.6 for CD45RA⁻ (CD45RA^low) cells, but 11.4 for CD45RA⁺ cells.

ELISPOT assays were then carried out on unsorted PBLs, due to an insufficiency of pure

15   cell populations. The number of positives for Melan-A peptide antigen was extremely low,

indicating high underestimation using the technique.

In the table which follows, the values for CD45RA⁻ (CD45RA^low) cells for HD 329, HD

604 and LAU 203 were normalized, because frequency values were below the lower limit of

detection.

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| | Melan-A specific CD8$^+$ T-cell frequency [10^{-5}] | | | | |
| | Tetramers | | LDA | | ELISPOT |
CD8$^+$ subset:	CD45RA$^+$	CD45RA$^-$ (CD45RA^{low})	CD45RA$^+$	CD45RA$^-$ (CD45RA^{low})	total
HD329	98 ± 11	7 ± 2	13 ± 0.2	0.3 ± 0.0	0.9 ± 0.9
HD604	190 ± 6	12 ± 3	9.7 ± 1.9	0.1 ± 0.0	0.0 ± 0.0
LAU 132	36	33	3.3 ± 0.4	9.1 ± 0.1	5.5 ± 5.6
LAU 203	100	4	13 ± 2.9	1.3 ± 0.1	0.0 ± 0.0

Example 37

Additional experiments were carried out to provide a better comparison of the sensitivity of the ELISPOT technique to tetramer staining. In these experiments, PBLs from the 10 healthy donors, and twelve of the thirteen melanoma patients were analyzed, using SEQ ID NOS: 3 and 5, staining assays and the ELISPOT assay.

Significant levels of spots specific for SEQ ID NO: 5 were found in all cases (over three times background), with deduced frequency correlating to the tetramer calculations. The ELISPOT assay did generally underestimate the frequency of CTL precursors specific for SEQ ID NO: 5.

On the other hand, SEQ ID NO: 3 specific spots were only detectable in one melanoma patient. The apparent frequency of SEQ ID NO: 3 specific, ELISPOT positive cells was 93 times lower than that obtained using tetramer staining. When some portion of the SEQ ID NO: 3 tetramer positive cells presented antigen experienced phenotype, frequency was less underestimated (12, and 4 fold, in two assays). This appears to show that efficient detection by ELISPOT is restricted to memory phenotype, antigen specific cells.

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Example 38

The fate of SEQ ID NO: 3 specific T cells in vivo over time was studied. In these experiments, blood samples were taken from a melanoma patient over a period of two years. To elaborate, the patient had presented primary skin melanoma of the lower limb. Inguinal lymph node dissection revealed that 4 of 6 lymph nodes were infiltrated by melanoma cells. The patient received isolated limb perfusion therapy (melphalan), and then adjuvant IFN alpha therapy, over 1.5 years. A second inguinal lymph node dissection showed that 15/16 lymph nodes were infiltrated with melanoma. The patient was tumor free for nearly two years. Over that period, he received immunizations of melanoma specific peptides (3-4 weekly in subcutaneous injections of 100μg of each of various peptides in PBS). The peptides corresponded to amino acids 26-35 of Melan-A (i.e., SEQ ID NO: 2), amino acids 1-9 of tyrosinase (i.e., MLLAVLYCLL, SEQ ID NO: 49), amino acids 368-378 of tyrosinase (i.e., SEQ ID NO: 4), amino acids 280-288 of gp 100, (YLLEPGPVTA, SEQ ID NO: 50) and amino acids 457-466 of tyrosinase (i.e., LLDGTATLRL; SEQ ID NO: 51). Five cycles of immunization were given, four of which included daily, subcutaneous administration of GM-CSF, starting 4 days before immunization, and covering the entire period of immunization.

Prior to the first immunization cycle, SEQ ID NO: 3 tetramer positive CD8$^+$ T cells of the patient (0.04% of total CD8$^+$ T cells), presented a naive, CD45RA$^+$ phenotype (The assay used is as described supra, using the same purification methods, anti-CD45RA Cy-Chrome labelled mAbs, and anti-CD28 FITC labelled mAbs).

One month after the end of the first injection, and until the end of the second cycle of immunization, half of the tetramer$^+$ cells presented antigen experienced, CD45RA$^-$(CD45RA^{low}) phenotype, accompanied by a small increase in frequency of SEQ ID NO: 3 positive cells (0.04%

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to 0.07%). During the second year, the CD45RA⁻(CD45RAˡᵒʷ) SEQ ID NO: 3 positive cells

gradually disappeared (dropping from 51% to 23%), while the frequency of total SEQ ID NO:

3 tetramer positive cells remained constant. The vast majority of these cells continually

displayed CD28 positive phenotypes.

5   **Example 39**

The experiments described herein were designed to obtain tumor antigen specific T cells

from tumor infiltrated lymph nodes ("TILN" hereafter). Single cell suspensions were prepared

from a TILN sample taken from a melanoma patient, in accordance with Romero, et al., J.

Immunol. 159: 2366-2374 (1997), incorporated by reference. The patient had been typed,

10   previously, as HLA-A⁺ 0201 positive. The single cells were cultured in medium (2 ml of

Iscove's Dulbecco medium, supplemented with Asn, Arg and Gln, 10% pooled, human A⁻ serum

and recombinant IL-2 (100 U/ml) and recombinant IL-7 (10ng/ml). After 16 days of culture, the

cells were analyzed as described supra, using tetramers containing SEQ ID NO: 3, mAbs to CD3

labelled with peridinin chlorophyll protein (PerCP), and mAbs to CD8, labelled with FITC. The

15   TILN were found to contain more than 90% CD3⁺/CD8⁺ cells. Of these, 19.4% were positive

for the tetramers, which is a high percentage.

Example 40

These experiments detail the expansion of the positive population described supra. First,

cells were sterile sorted into positive cells, using standard methods. The positive and negative

20   cells were then cultured for 2 weeks in the presence of irradiated, allogeneic PBMCs and

phytohemagglutinin, as described supra. Following the culture, the cells were retested, using

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tetramers and the CD8 specific mAb described supra. The positive portion contained 94.4%

tetramer positive cells, and the negative portion, 1.1% positives.

Example 41

Following the experiments described supra, the expanded populations of positive and

5   negative cells, as well as TILN from LAU 203 were tested in a ^{51}Cr release assay, as described

supra, together with the peptide of SEQ ID NO:2, which was added at 1μM, or without it, using

T2 cells labelled with the ^{51}Cr as a target.

No cytotoxicity was observed without the peptide. The tetramer positive population

showed enhanced antigen specific cytolytic activity as compared to unfractionated TILN, which

10   was proportional to the level of enrichment. A low level of cytolysis was detected in the negative

cells, possibly resulting from a small percentage of tetramer positive T cells in the culture.

Example 42

These experiments were designed to compare the fine specificities of the unsorted, TILN

population and the tetramer positive cells described supra. This was done by carrying out ^{51}Cr

15   release assays, as described supra, using T2 cells which had been incubated with varying

concentrations of different peptides, in 50 μl samples. The peptides tested were SEQ ID NOS:

1, 2, 3, 37 and 38. The T2 cells were incubated with the peptides for 15 minutes at room

temperature before adding either unsorted TILN population cells or the sorted, tetramer positive

cells, at an effector/target ratio of 10/1. Chromium release was measured after 4 hours at 37°C.

20   The results are presented in figures 7A and 7B, where figure 7A shows the results obtained using

the unsorted TILN population and 7B the sorted population. The two populations showed

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similar lysis patterns. Parental decapeptide (SEQ ID NO: 1) was recognized about 5 fold more efficiently than the parental nonapeptide (SEQ ID NO: 1), but decapeptide SEQ ID NO: 3 was recognized much more efficiently, with half maximal lysis being obtained at low peptide concentrations, i.e., 10^{-10} to 10^{-11} M.

5   These experiments show nearly identical fine specificity in sorted and unsorted TILN, showing that the tetramer positive, sorted cell population can be considered a polyclonal, monospecific T cells population, representative of the total T cell population which is specific for the antigen/MHC complex of interest.

Example 43

10   T cell receptor down regulation is one of the earliest T cell activation events induced upon antigen recognition and subsequent TCR triggering. The tetramer sorted population discussed supra was used to study this.

Specifically, T2 cells were pulsed for 1 hour at 37°C with varying concentrations of peptides, and were then washed, twice, to avoid peptide autopresentation by T cells. Samples

15   (1×10^5) of the T cells were then stimulated by peptide pulsed T2 cells (2×10^5). After 6 hours of incubation at 37°C, the cells were stained for CD3 using a fluorescent labelled monoclonal antibody, and were then analyzed, via standard methods. The parental Melan-A peptides (SEQ ID NOS: 1 and 2) induced only weak TCR down regulation even at the high peptide doses that were efficient in target CTL sensitization; however, maximal TCR down regulation was induced

20   by SEQ ID NO: 3.

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Example 44

These experiments describe studies of Ca^{2+} responses. These responses occur a few seconds after T cell receptors are engaged. Essentially, when T cells are activated, calcium enters the cells, and can be measured. T cells were loaded with a commercially available fluorescent Ca^{2+} indicator, Indo-1 AM, described by Grynkiewicz, et al. J. Biol. Chem. 260(6): 3440-3450 (1985) following Valitutti, et al., J. Exp. Med. 183: 1917-1921 (1996), both of which are incorporated by reference. As a positive control, a CTL clone was used which had been derived from a normal HLA-A2 positive donor, PBMC's of whom had been stimulated, in vitro, with SEQ ID NO: 2. Samples of this CTL were combined with T2 cells that had been pulsed, for 2 hours at 37°C, with serial dilutions of SEQ ID NO: 2 or SEQ ID NO: 3. Cells were centrifuged for one minute at 1500 x g, incubated for 1 minute at 37°C, and then resuspended and analyzed by flow cytometry.

SEQ ID NO: 3 triggered a full Ca^{2+} response, but the CTL clone referred to supra required a concentration of about 1 ng/ml. In contrast, the polyclonal, tetramer positive cells discussed supra were triggered for Ca^{2+} at a broader range of peptide analogue concentrations. Some cells even reacted at the lowest peptide concentration, i.e., 10^{-3} ng/ml. Further, at the maximal peptide concentrations tested, SEQ ID NO: 2 triggered Ca^{2+} influx in only 60% of the cells of the CTL clone, and 90% of the polyclonal population. In contrast, SEQ ID NO: 3 mobilized Ca^{2+} in nearly 100% of the CTL sample.

The data also showed that almost 50% of TCR down-regulation was required to attain a full Ca^{2+} response. SEQ ID NO: 3 also induced a more rapid Ca^{2+} response as compared to the parental peptide.

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The observation that 90% of the tetramer positive population increased Ca^{2+} in response to the parental decapeptide confirmed that the tetramer positive population was highly specific for the parental peptide.

Example 45

5   In addition to the early activation events described in examples 43 and 44, late activation events were also studied, i.e., cytokine synthesis, and induction of cytolytic effector cell function. The former was done via intracellular staining of cytokines, which is described by Jung, et al., J. Immunol. Meth. 159:197-207 (1993), as a very sensitive method for determining cytokine response at the single cell level. Labelled monoclonal antibodies against TNF-alpha, INF

10   gamma, GM-CSF, and IL-4 were used. All mAbs were labelled with phycoerythrin. In brief, 5×10^4 T cells were stained with different mAbs, at concentrations of 5mg/ml, for 30 minutes at room temperature. Dilutions and washes were carried out using PBS 10.1%, containing 0.1% saponin. The saponin permeabilizes the cell, allowing entry of the mAbs. The production of each of these cytokines by the CTL line described supra, and the polyclonal, tetramer positive

15   population, in response to different peptide concentrations were measured. Peptides defined by SEQ ID NOS: 1, 2 and 3 were tested. The results are presented in figure 8. "Clone 1.13" is the CTL clone referred to supra.

None of the peptides stimulated the entire monoclonal or polyclonal population, which is consistent with prior reports that a fraction of CTLs is refractory to cytokine production. SEQ

20   ID NOS: 1 and 2 only stimulated small fractions of the CTLs (e.g., only 30% of the cells of the CTL clone produced TNF-alpha at the highest concentrations of these peptides). Similarly, 45% and 10% of the polyclonal population produced TNF-alpha in response to SEQ ID NO: 1 and

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SEQ ID NO: 2, respectively. In contrast, SEQ ID NO: 3 stimulated the totality of CTLs able to

produce cytokines, with a plateau being reached at 1 μM, for each population. It is interesting

to note that 45% of the polyclonal population produced some TNF-alpha, while 90% of these

cells increased intracellular Ca^{2+} concentration at the highest concentration of SEQ ID NOS: 1

5   and 2 used.

The percentage of the population which produced IL-2 and IL-4 was always less than the

percentage producing TNF-alpha, regardless of the peptide concentrations used.

The polyclonal population was distinguishable from the monoclonal population in that

it had a greater capacity to produce IFN-gamma, and was incapable of producing IL-4, which is

10   consistent with prior observations.

Double staining experiments were carried out on both populations, following stimulation

with 10 μM of SEQ ID NOS: 1, 2, and 3. The double stainings were IFN-gamma and TNF-

alpha, and IFN-gamma and IL-2. SEQ ID NOS: 1 and 2 stimulated low fractions of these cells

to produce the cytokines; however, when SEQ ID NO: 3 was used, both the percentages of cells

15   producing the cytokines, and the amount of cytokine produced per cell were increased.

Most cells produced TNF-alpha and IFN-gamma, while only a fraction of IFN-gamma

positive cells produced IL-2. More importantly, however, the fraction of IL-2 producing cells

and the amount of IL-2 produced on a per cell basis were highly increased after stimulation with

SEQ ID NO: 3.

20   **Example 46**

It is known that full CTL activation is associated with cell division and induction of

cytolytic effector function. Such functions require strongly agonistic peptides, and/or higher

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peptide concentrations, in order to maximize induction of expansion and cytolytic effector

function, as compared to target sensitization, or induction of cytokine synthesis. In the case of

the peptides being studied herein, the low potential of SEQ ID NOS: 1 and 2 to induce cytokine

production by specific CTLs may mean that these peptides are inefficient activators of cytolytic

5   effector function. These experiments were designed to test this.

Peripheral blood lymphocytes of patent LAU 203 were stimulated with syngeneic

stimulator cells (PBMCs) that had been pulsed for 2 hours at 37°C in serum free medium together

with 1μM of one of SEQ ID NOS: 1, 2, 3, 38 or 39, and 3 ug/ml of human β2-microglobulin.

These peptide pulsed PBMCs were washed, twice, irradiated (3000 rads), and then adjusted to

10   an appropriate volume before adding to the responder cells. In addition to the stimulator cells,

IL-2 (10 U/ml), and IL-7 (10 ng/ml), were added during the first two stimulation cycles (week

1 and week 2), and then IL-2 alone (100 U/ml) was added during the third week.

After 3 weeks of incubation, the PBMCs were analyzed via flow cytometry. The results

showed that cultures that had been stimulated with SEQ ID NO: 1 or 2 had 1.7% and 3.5%

15   tetramer positive cells, while those populations stimulated with SEQ ID NOS: 3, 38 and 39 had

11.2% (SEQ ID NO: 3), 19.7% (SEQ ID NO: 38), and 37.0% (SEQ 10 NO: 39) percent

positives.

Cytotoxicity was also tested, in ^{51}Cr release assays as described supra. The cytotoxicity

induced by SEQ ID NOS: 1 and 2 was weak, while analogue peptides induced strong cytotoxicity

20   against target cells labelled with parent peptides SEQ ID NOS: 1 and 2, and autologous tumor

cells. This strong cytotoxicity was caused by high frequency of antigen specific cells in the

culture, and strong activation of these cells.

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Example 47

Experiments were carried out to study inhibition of CTL function. More particularly, notwithstanding the observations described in the previous examples, it is known that in vivo T cell activity does not protect most cancer patients.

There has been some speculation that cytolytic function is inhibited through the activity of natural killer ("NK" hereafter) receptors. Two families of these have been observed, i.e., type I transmembrane proteins, which belong to the immunoglobulin (Ig) superfamily, such as p58.2 (Moretta, et al., J. Exp. Med. 182:875-884 (1995)) and ILT2 (Colonna, et al., J. Exp. Med. 186:1809-1818 (1997)), and those which are type II transmembrane proteins which contain a C-type lectin domain, such as the CD94/NKG2 heterodimer. (Maretta, et al., J. Exp. Med. 180:545-555 (1994)). The experiments in this example were designed to determine whether NK receptors may interfere with in vivo tumor specific responses.

First, $CD8^+$ cells were examined for NK receptor phenotype. To do this, blood samples and lymph nodes were obtained from patients with advanced stage malignant melanoma, using standard techniques. (The patients had been typed as HLA-A*0201 positive via flow cytometry of PBMCs, using mAb BB7.2, described supra.)

PBLs were separated from blood samples taken from patients who suffered from vitiligo as well as melanoma via centrifugation over Ficoll-Paque, washed 3 times, and then cryopreserved in RPMI 1640, 40% fetal calf serum, and 10% DMSO. Vials containing from 5-$10x10^6$ cells were stored in liquid nitrogen.

Lymph nodes were collected by surgical dissection, dissociated into single cell suspensions in sterile RPMI 1640 supplemented with 10% fetal calf serum, washed and cyropreserved, as described supra. Aliquots of cells were placed in 24 well tissue culture plates

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in 2ml of Iscove's Dulbecco medium, supplemented with 0.55 mM Arg, 0.24 mM Asn, 1.5 mM Gln, 10% pooled human A$^+$ serum, recombinant human IL-2 (100 U/ml), and recombinant human IL-7 (10ng/ml).

5   Both CD8$^+$ cells and the cells taken from the lymph nodes were contacted with monoclonal antibodies against p58.2/CD158b, CD94, CD94/NKG2A, and ILT2, each of which is an NK receptor, and tetramers of SEQ ID NO:3 labelled with phycoerythrin.

The TILNs contained high percentages of CD8$^+$ tetramer$^-$ cells (1.3-5.1%). In one of the samples analyzed, these were largely negative for p58.2 and ILT2, but were highly positive for CD94 and CD94/NKG2A. A sample of TILN from another patient expressed some ILT2, but

10   low levels of the others. Yet a third sample expressed ILT2, CD94, and CD94/NKG2A.

The PBLs were found to contain from 0.10 to 0.17% CD8$^+$ tetramer$^-$ cells. One sample was negative for all of the receptors. The CD8$^+$ tetramer$^+$ cells of a second sample were almost all positive for CD94 and CD94/NKG2A, and about half expressed ILT2. A third sample of PBLs showed a _low_ percentage of NK receptor positive cells.

15   **Example 48**

The relatively high frequency of tumor specific CTLs in the vitiligo patients permitted investigation of the _in vivo_ phenotype of these cells. Fluorochrome labelled anti-CD45RA mAbs were used, in an assay as described _supra_. Two samples had high levels of CD45RA positive cells, which are indicative of naive T cells, while reduced levels were found in a third.

20   Additional assays were carried out using fluorochrome labelled mAbs against CD28 and adhesion molecule CD57, since downregulation of CD28 and upregulation of CD57 have been recognized in activated effector CTLs.

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It was found that samples taken from the one vitiligo subject, described supra, with a high percentage of NK receptor expressing CTLs were predominantly CD28⁻ and CD57⁺, while the other two subjects were CD28⁺ and CD57⁻.

Example 49

5   Given that the NK receptors had been typed, it was important to determine if they were functional. Previously, it had been shown that antigen specific, T cell cytotoxicity can be inhibited through the triggering of the receptor CD94/NKG2A (Noppen, et al., Eur. J. Immunol. 28:1134-1142 (1998)); or p58.2 (Phillips, et al., Science 268: 403-405 (1995); Ikeda, et al., Immunity 6: 199-208 (1997), Bakker, et al., J. Immunol. 160:5239-5245 (1998)). Experiments

10   were developed, and are described herein, to investigate cytotoxicity of cells ex vivo, directly after withdrawal from the patient. PBLs were sorted from two different samples, and sorted via FACS, using methodologies described supra, using anti CD8 and either anti p58.2 or anti CD94/NKG 2A antibodies. The sorted suspensions were cultured, overnight, in Iscove Dulbecco's medium supplemented with 0.55 mM Arg, 0.24 mM Asn, 1.5mM Gln, 10% pooled

15   human A⁺ serum, and recombinant human IL-2 (10 U/ml).

Cytolytic activity was then tested in anti-CD3 mAb redirected ⁵¹Cr release assays. In these assays, Fcγ-receptor expressing P815 mastocytoma cells were radiolabelled with Na⁵¹CrO₄ for 1 hour at 37°C, at 5% CO_2. After washing, 10^3 P815 target cells were coincubated with PBLs which were either CD94/NKG2A positive or CD94/NKG2A negative, at varying effector: target

20   levels, together with varying concentrations of anti-CD3 mAbs. The anti-CD3 mAbs cross link CD3 molecules on T cells to the Fcγ-receptors, leading to the lysis. Samples were incubated for

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4 hours at 37°C, after which supernatant were collected and radioactivity measured. To determine the role of the receptor, anti CD94/NKG2A antibody was either added, or withheld. As a control, anti-CD19 antibodies were used.

The results are shown in figure 9. Squares represent tests carried out using anti CD94/NKG2A antibody, and triangles, the control, anti-CD19 antibody.

In the presence of the anti-CD94/NKG2A antibody, the cells positive for this receptor showed reduced killing, as compared to killing in the presence of anti CD19 antibody, indicating that the antibody stimulated the NK receptor, i.e., the CD94/NKG2A receptor. In contrast, cytolytic activity of those $CD8^+$ cells negative for this receptor were not reduced in the presence of the antibody. Further, the NK receptor positive population showed stronger cytolytic activity, as compared to the NK receptor negative population.

Example 50

Phenotypic analysis of various samples revealed one which had an unusually high frequency of $\alpha\beta TCR^+$, CD28 negative cells which expressed p58.2. These cells provided a singular opportunity to test functional consequences of p58.2 binding to its natural ligand HLA-Cw3. As in example 49, cells of this sample were sorted via FACS, into $CD8^+$ $p58.2^+$ and $CD8^+$ $p58.2^-$ populations, and were tested as described supra, but without any antibodies. P815 cells were used, as were P815 cells transfected to express HLA-Cw3, using known methods.

The P815 cells were killed efficiently by both p58.2 positive and negative cells at effector: target ratios of 5.1; however, lysis of transfected cells was reduced markedly, indicating inhibition of lysis via interaction of p58.2 with HLA-Cw3. In the absence of CD3 specific antibodies, killing was below 2%.

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The foregoing examples describe aspects of the invention, which is a multicomponent complex useful, e.g., in isolating cytolytic T cells specific for a particular target, from a sample. The complex comprises a first binding partner and a second binding partner, wherein the first and second binding partner are specific for each other. These can be, e.g., avidin or streptavidin and biotin, an antibody or a binding portion of an antibody specific to biotin, and so forth. The key feature is that the second binding partner must be bound to a plurality of complexes of an MHC molecule, a ß2 microglobulin molecule and a peptide which binds specifically to said MHC molecule, and the multicomponent complex must be labelled. The MHC molecules are preferably HLA molecules, such as HLA-A2 molecules. The examples all show HLA-A*0201, however, it will be understood by the artisan of ordinary skill that any HLA molecule could be used. With respect to the peptide of interest, many references, including review articles, U.S. and non-U.S. patents, and so forth describe peptides beyond SEQ ID NOS: 2-5 and their binding partner HLA molecule. All are encompassed by the invention. Exemplary peptides and their HLA molecule partners are presented later in this application.

Preferably, the second binding partner is biotin, but it may also be, e.g., an antibody which is specific for a component of the HLA/ß2 microglobulin/peptide complex, such as an HLA specific antibody, or a ß2 microglobulin specific antibody. Similarly, the first binding partner may be e.g., recombinant or naturally occurring protein L, recombinant or naturally occurring protein A, or even a second antibody. The complex can be in soluble form, or bound, e.g., to a removable solid phase, such as a magnetic bead.

The number of HLA/ß2 microglobulin/peptide complexes in the large molecule of the invention may vary. It comprises at least two complexes, and preferably at least four, but more may be present as well.

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The complex of binding partners and HLA/ß2 microglobulin/peptide may be labelled, using any of the labels known to the art. Examples of fluorescent labels are given supra. Enzymatic labels, such as alkaline phosphatase, metal particles, colored plastics made of synthetic materials, radioactive labels, etc., may all be used.

5   A third binding partner may also be used, which binds, specifically, to the first binding partner. For example, if the first binding partner is streptavidin, and the second binding partner is biotin, then the third binding partner may be a streptavidin specific antibody. When three or more binding partners are used, the label referred to supra may be attached to any of the binding partners so long as engagement with the HLA/ß2 microglobulin peptide complexes is not

10   impaired.

The complexes may be used, e.g., to identify or to isolate cytolytic T cells present in a sample, where these cells are specific for the HLA/ß2 microglobulin/peptide complex. As the examples show, such cytolytic T cells bind to the immunocomplexes of the invention. In a preferred embodiment, the sample being tested is treated with a reactant which specifically binds

15   to a cytolytic T cell, wherein said label provides a detectable signal. The sample, including labelled CTLs, is then contacted to the complex, where it binds, and can be separated via any of the standard, well known approaches to cell separation. Preferably, FACS is used, but other separation methodologies will be known to the skilled artisan as well. The peptide used is left to the needs of the skilled artisan, and will depend, e.g., on the nature of the specific MHC

20   system under consideration, a table of exemplary, but no means the only, peptides for which CTLs are known, follows. These are also set forth as SEQ ID NOS: 6-36.

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	Gene	MHC	Peptide	SEQ ID
	MAGE-1	HLA-A1	EADPTGHSY	6
5		HLA-Cw16	SAYGEPRKL	7
	MAGE-3	HLA-A1	EVDPIGHLY	8
		HLA-A2	FLWGPRALV	9
		HLA-B44	MEVDPIGHLY	10
	BAGE	HLA-Cw16	AARAVFLAL	11
10	GAGE-1,2	HLA-Cw16	YRPRPRRY	12
	RAGE	HLA-B7	SPSSNRIRNT	13
	GntV	HLA-A2	VLPDVFIRC(V)	14
	MUM-1	HLA-B44	EEKLIVVLF	15
			EEKLSVVLF	16
15	CDK4	HLA-A2	ACDPHSGHFV	17
			ARDPHSGHFV	18
	ß-catenin	HLA-A24	SYLDSGIHF	19
			SYLDSGIHS	20
	Tyrosinase	HLA-A2	MLLAVLYCL	21
20		HLA-A2	YMNGTMSQV	22
		HLA-A24	AFLPWHRLF	23
		HLA-B44	SEIWRDIDF	24
		HLA-B44	YEIWRDIDG	25
		HLA-DR4	QNILLSNAPLGPGFP	26
25		HLA-DR4	DYSYLQDSDPDSFQD	27
	Melan-A[Mart-1]	HLA-A2	(E)AAGIGILTV	28
		HLA-A2	ILTVILGVL	29
	gp100[Pmel117]	HLA-A2	KTWGQYWQV	30
		HLA-A2	ITDQVPFSV	31
30		HLA-A2	YLEPGPVTA	32
		HLA-A2	LLDGTATLRL	33

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	HLA-A2	VLYRYGSFSV	34
DAGE	HLA-A24	LYVDSLFFL	35
MAGE-6	HLA-Cw16	KISGGPRISYPL	36

Additional peptides may be found, e.g., in U.S. patent application Serial Nos. 08/672,351, 08/718,964, 08/530,569, and 08/880,963, all of which are incorporated by reference, as well as U.S. Patent No. 5,821,122, also incorporated by reference.

Additionally, the method can be used to monitor the status of tumors, following administration of a particular therapeutic agent, such as a vaccine. Further, since the methodology can be used to identify cytolytic T cell precursors, as shown, supra, one can identify candidates for potential therapies by determining if they possess the relevant T cell precursors.

Also a part of the invention is the use of tetramers as described, in conjunction with other steps, to yield populations of T cells with desired features. These include specific phenotypes, such as phenotypes associated with antigen experienced, or memory cells, or naive cells, and so forth. Such populations can be cultured, in the absence of peptides, to yield cell populations which can be used, e.g., diagnostically or therapeutically. This culturing can be carried out, e.g., with a mitogen, such as phytohemagluttinin, and without peptides.

The invention also involves methods for obtaining desired T cells via in vivo recruitment. The examples show, e.g., that one can inject a peptide of interest into a subject, e.g., subcutaneously, leading to recruitment of T cells. In turn, the T cells can be separated, and cultured, in the same manner described supra.

The invention also involves the ability to improve the lytic activity of a T cell population. As has been shown, one can assay a population of T cells, to determine if these T cells express NK receptors. Expression of said receptors allows one to gauge the lytic potential of the T cell

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population involved. An antibody assay is one way to do this, but hybridization assays, and other approaches may also be used.

Once the NK receptor expression levels are determined, one can improve the lytic ability of the population by adding NK receptor inhibitors, such as soluble, MHC molecules like soluble HLA-A*0201 or HLA-Cw*3 molecules, or agents which permit the T cell to function notwithstanding expression of NK receptors. An example of such an agent, as was shown, is an anti-CD3 antibody.

Other aspects of the invention will be clear to the skilled artisan, and need not be elaborated further.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

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Claims

1.   An isolated complex comprising a first binding partner and a second binding partner which bind to each other, at least one of which is labelled to provide a detectable signal, and a plurality of immune complexes comprising an MHC molecule, a ß2 microglobulin molecule, and a peptide which specifically binds with said MHC molecule.

2.   The isolated complex of claim 1, wherein said second binding partner is biotin.

3.   The isolated complex of claim 1, wherein said first binding partner is avidin or streptavidin.

4.   The isolated complex of claim 1, comprising at least four of said immune complexes.

5.   The isolated complex of claim 4, wherein said immune complexes are identical to each other.

6.   The isolated complex of claim 1, wherein said complex is labelled with a fluorescent or enzymatic label.

7.   The isolated complex of claim 1, wherein said immune complex is a complex found on surfaces of cancer cells.

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8.   The isolated complex of claim 1, wherein said first binding partner is an antibody or a

        binding fragment of an antibody.

9.   The isolated complex of claim 1, wherein said second binding partner is an antibody.

10.   The isolated complex of claim 1, further comprising a third binding partner which

        specifically binds to said first binding partner.

11.   The isolated complex of claim 10, wherein said third binding partner is an antibody.

12.   The isolated complex of claim 1, further comprising a solid phase to which said isolated

        complex is bound.

13.   The isolated complex of claim 1, wherein said solid phase is a magnetic bead.

14.   A method for determining presence of cytolytic T cells specific for an immune complex

        of an HLA molecule, a ß2 microglobulin molecule and a peptide, comprising contacting

        a T cell containing sample with the isolated complex of claim 1, and determining binding

        of cytolytic T cells to said isolated complex as a determination of said cytolytic T cells

        in said sample.

15.   The method of claim 14, further comprising labelling said cytolytic T cells with a

        detectable label which specifically binds to cytolytic T cells.

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16.   The method of claim 15, wherein said label which specifically binds to cytolytic T cells is an anti-CD8, anti-CD28, anti-CD45RO, or antiCD45RA antibody.

17.   A method for isolating cytolytic T cells which specifically bind to a complex of an HLA molecule, a ß2 microglobulin molecule, and a peptide, comprising contacting a sample containing said cytolytic T cells with the isolated complex of claim 1 to bind cytolytic T cells thereto, separating said isolated complex from said sample, and removing any cytolytic T cells bound thereto.

18.   A method for monitoring status of a tumor, comprising contacting a T cell containing sample from a subject with a tumor with the multimeric complex of claim 1 at a first point in time, repeating contact at a second point in time, and determining variation in T cell type or number at said second point in time as compared to T cell type or number at said first point in time as a determination of tumor status.

19.   The method of claim 18, further comprising administering a therapeutic agent to said subject after said first point in time and before said second point in time.

20.   A method for identifying a candidate for treatment of a condition characterized by a T cell response to said condition, comprising contacting a T cell containing sample from said candidate with the multimeric complex of claim 1, determining any cytolytic T cell precursors which bind thereto, and determining whether any of said cytolytic T cell precursors are of a type sufficient to alleviate said condition.

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21.   A method for preparing a population of cytolytic T cells specific for a complex of an

MHC molecule and a peptide, comprising:

        (a)   contacting a sample containing T cells specific for said complex with the

complex of claim 1, under conditions favoring binding of said T cells to said complex,

        (b)   removing said T cells, and

        (c)   culturing said T cells in a medium which contains no peptides.

22.   The method of claim 21, wherein said complex is a tetramer.

23.   The method of claim 22, wherein said tetramer comprises four MHC molecules,

and four peptide molecules, each of which is complexed with one of said four

MHC molecules.

24.   The method of claim 23, wherein said four peptide molecules are tumor rejection

antigens.

25.   The method of claim 21, further comprising adding an amount of said peptide to said

sample prior to contacting with said tetramer, for a time sufficient to stimulate T cells in

said sample.

26.   The method of claim 21, wherein said medium contains a mitogen.

27.   The method of claim 26, wherein said mitogen is phytohemaglutinin.

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28.   An isolated peptide, the amino acid sequence of which consists of an amino acid

sequence set forth in one of SEQ ID NOS: 39-47.

29.   The isolated peptide of claim 28, the amino acid sequence of which is set forth at SEQ

ID NO: 41, 43, 45 or 46.

30.   The isolated complex of claim 4, comprising 4 molecules of SEQ ID NO: 1, 2 , 3 or 4,

each of which is complexed to an HLA-A* 0201 molecule.

31.   The method of claim 25, wherein said peptide is the peptide of SEQ ID NO: 4.

32.   The isolated complex of claim 4, comprising 4 molecules of SEQ ID NO: 9, each of

which is complexed to an HLA-A* 0201 molecule.

33.   The method of claim 25, wherein said peptide is the peptide of SEQ ID NO: 9.

34.   A method for obtaining a population of T cells, comprising, injecting skin of a subject

with an amount of a peptide sufficient to stimulate recruitment of cytolytic T cells to the

site of injection, taking a skin biopsy from said site of injection, separating any

lymphocytes from said biopsy, isolating a subset of lymphocytes specific for complexes

of said peptide and an MHC molecule from said lymphocytes, by contacting the

lymphocytes with the isolated complex of claim 4, and stimulating said subset of

lymphocytes.

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35.   The method of claim 34, wherein said subject is afflicted with cancer.

36.   The method of claim 34, further comprising separating said subset of lymphocytes into naive cells and memory cells.

37.   The method of claim 34, comprising stimulating said subset of lymphocytes with an amount of the peptide used for injection.

38.   The method of claim 34, comprising stimulating said subset of lymphocytes in the absence of any peptide and in the presence of a mitogen.

39.   The method of claim 34, further comprising separating said subset of lymphocytes by determining cell surface markers thereon.

40.   A method for stimulating production of T cells of interest, comprising contacting a cell sample believed to contain T cells with the isolated complex of claim 4, separating any T cells which bind to said complex from said samples, phenotyping said T cells based upon cell surface markers, separating a population of said T cells which possess cell surface markers of interest, and stimulating proliferation of said population.

41.   The method of claim 40, wherein said cell surface molecules of interest are markers which indicate that said T cells are antigen experienced.

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42.   A method for assessing lytic ability of a T cell population, comprising assaying said T

cell population to determine NK receptor expression by said T cell population.

43.   The method of claim 42, comprising assaying said T cell population with an antibody

which specifically binds to an NK receptor.

44.   The method of claim 43, wherein said NK receptor is p58.2 or CD94/NKG2A.

45.   A method for improving lytic activity of a T cell population, comprising adding an agent

to said T cell population which inhibits activity of any NK receptors expressed by said

T cell population.

46.   The method of claim 45, wherein said agent is a soluble MHC molecule or a portion of

an MHC molecule which binds to said NK receptor.

47.   The method of claim 46, wherein said agent is a soluble HLA-A2 or HLA-Cw*3

molecule.

48.   A method for improving lytic activity of a T cell population, at least a portion of which

express NK receptors, comprising adding an agent to said T cell population which

facilitates binding of a T cell to its target.

49.   The method of claim 48, wherein said agent is a cross-linking antibody.

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50. The method of claim 49, wherein said cross-linking antibody is an anti-CD3 antibody.

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FIG. 1A
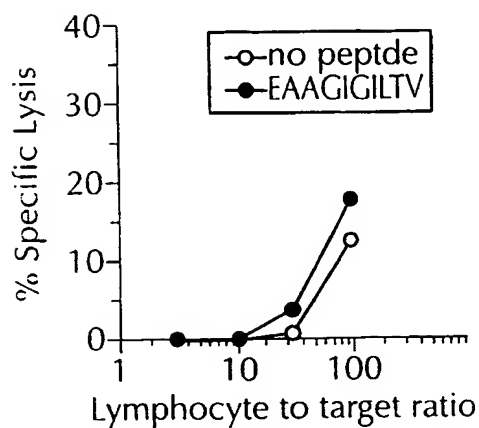
FIG. 1B

FIG. 1C

FIG. 1D

FIG. 1E

FIG. 1F

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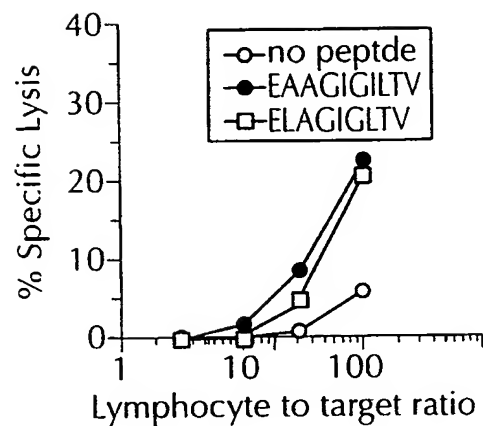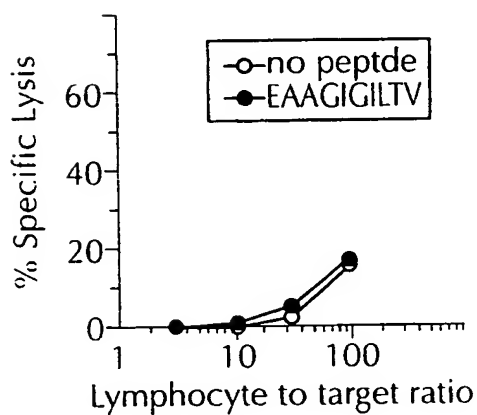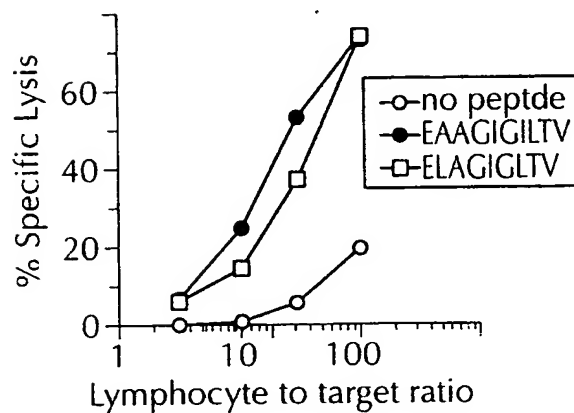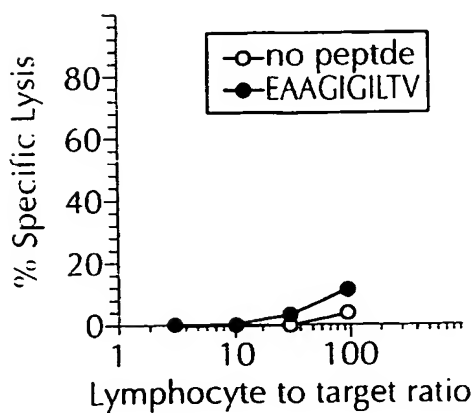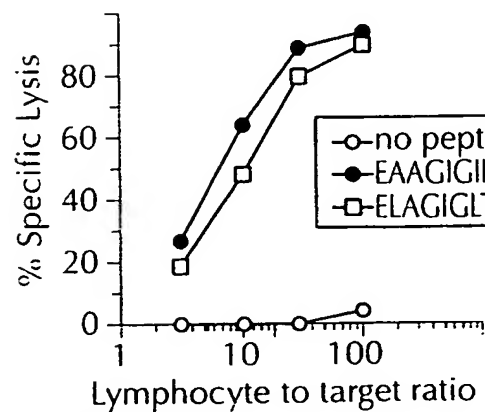
FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

FIG. 2E

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FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

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FIG. 3E

FIG. 4

FIG. 5A

FIG. 5B

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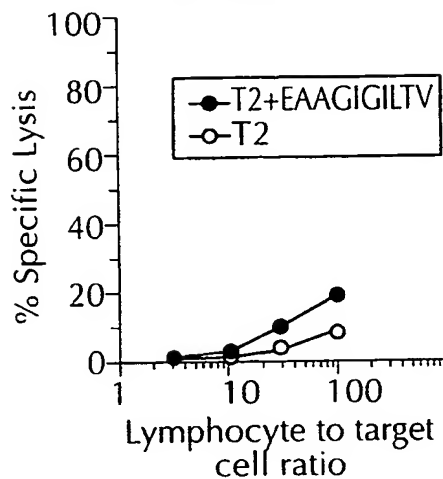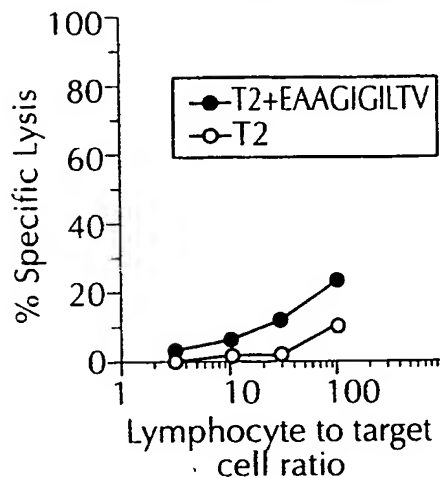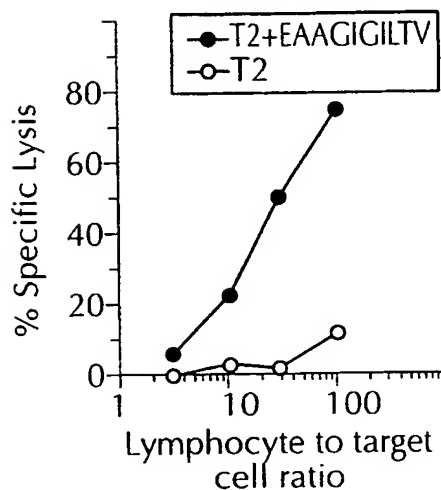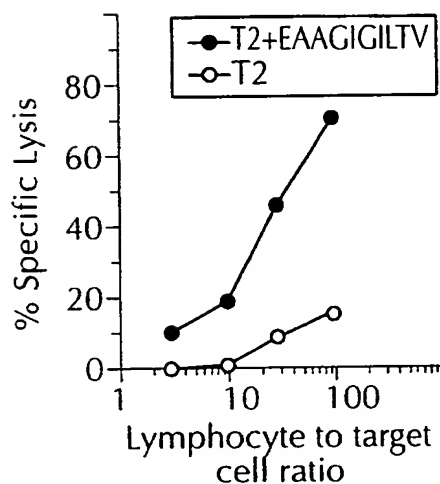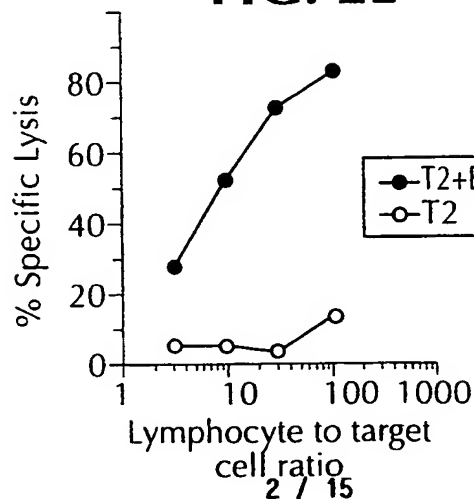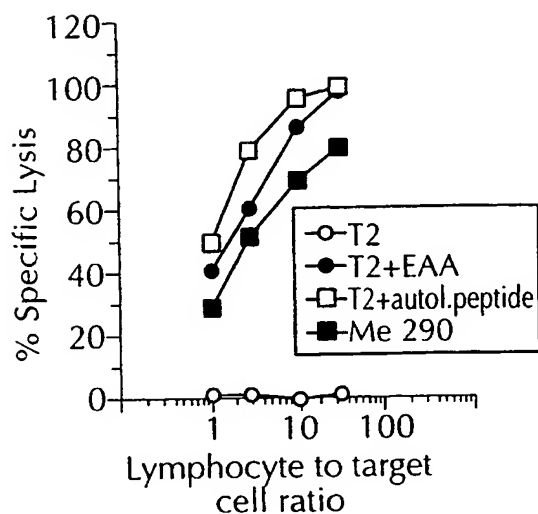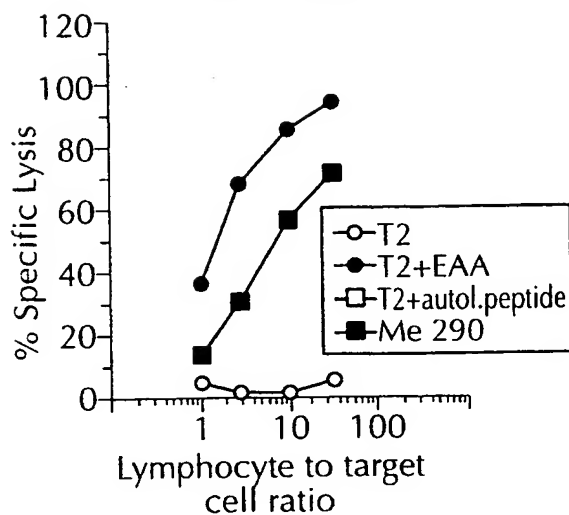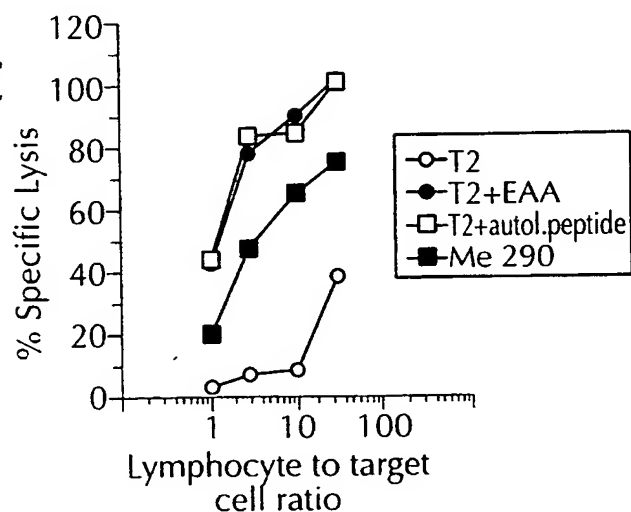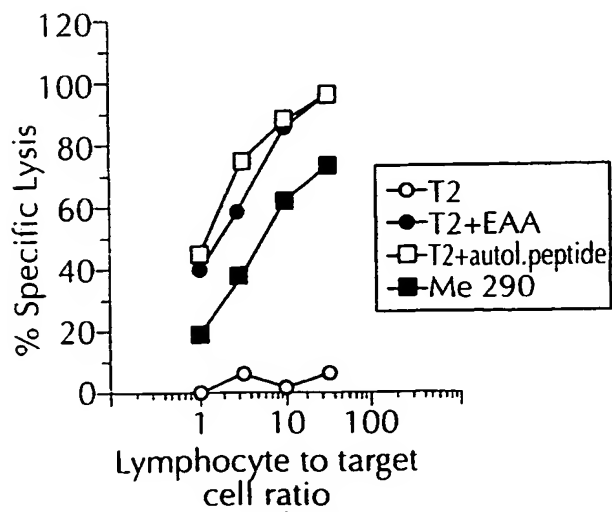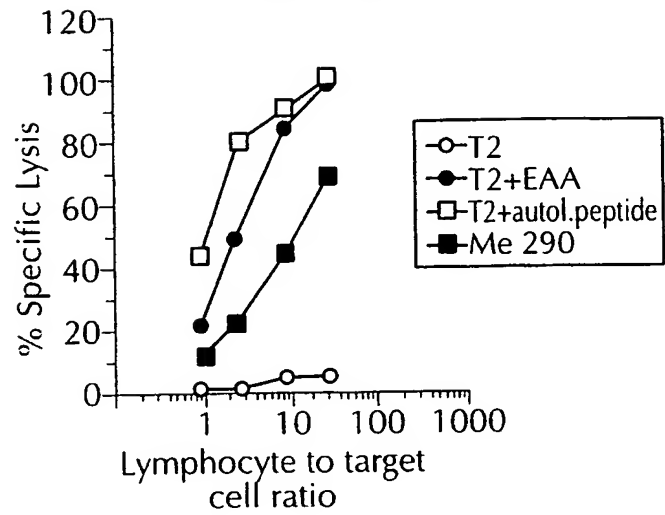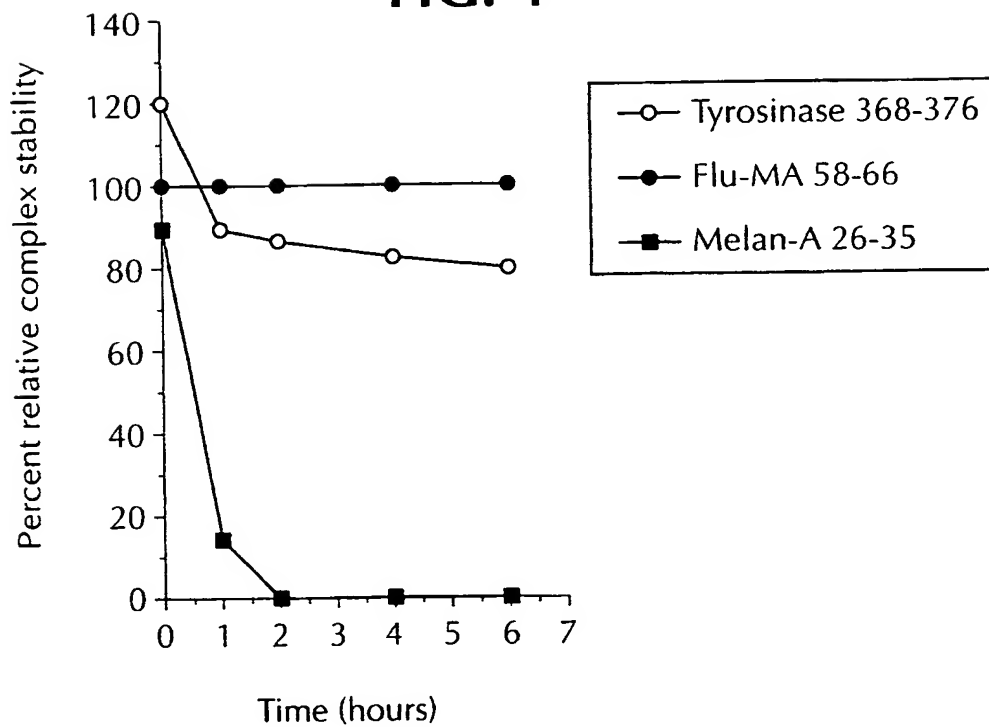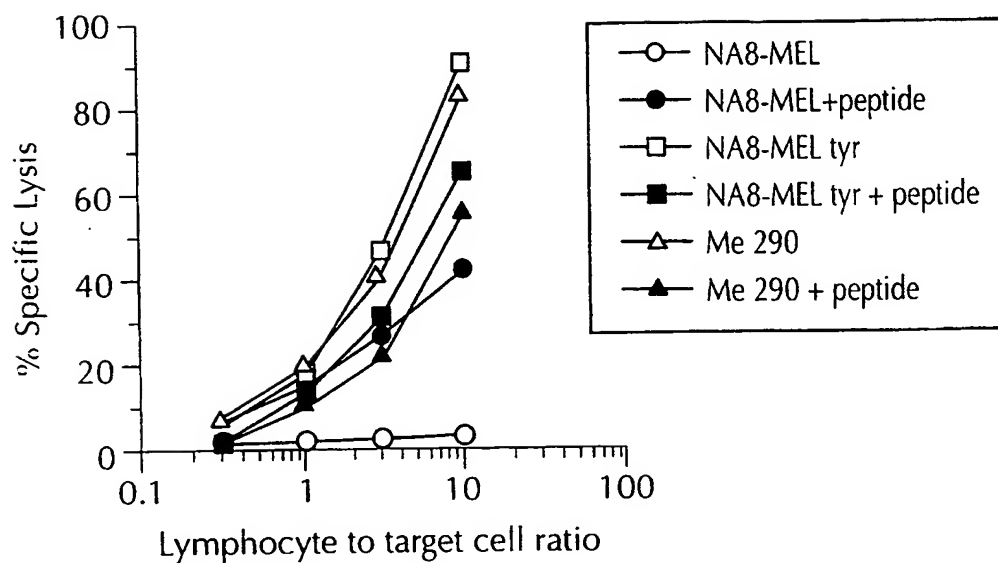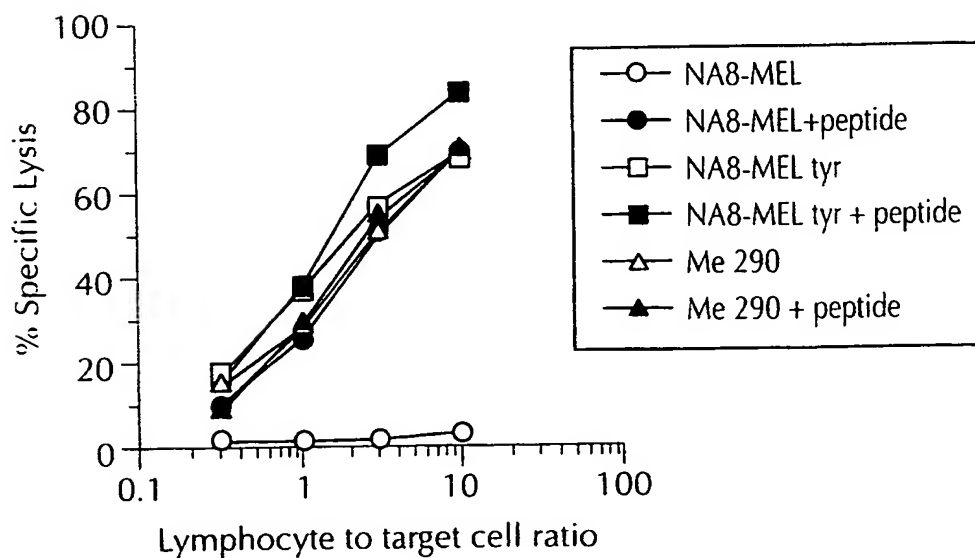
FIG. 5C
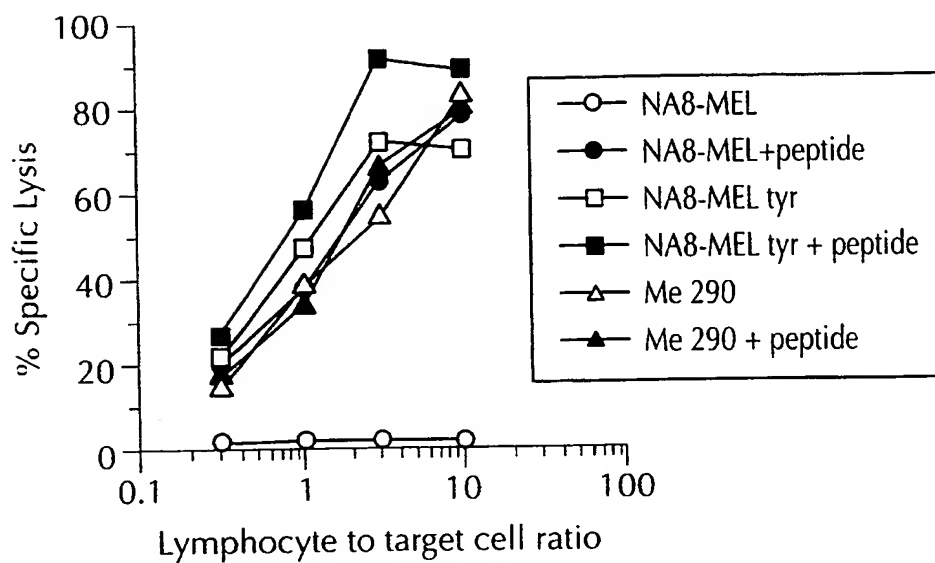
FIG. 5D
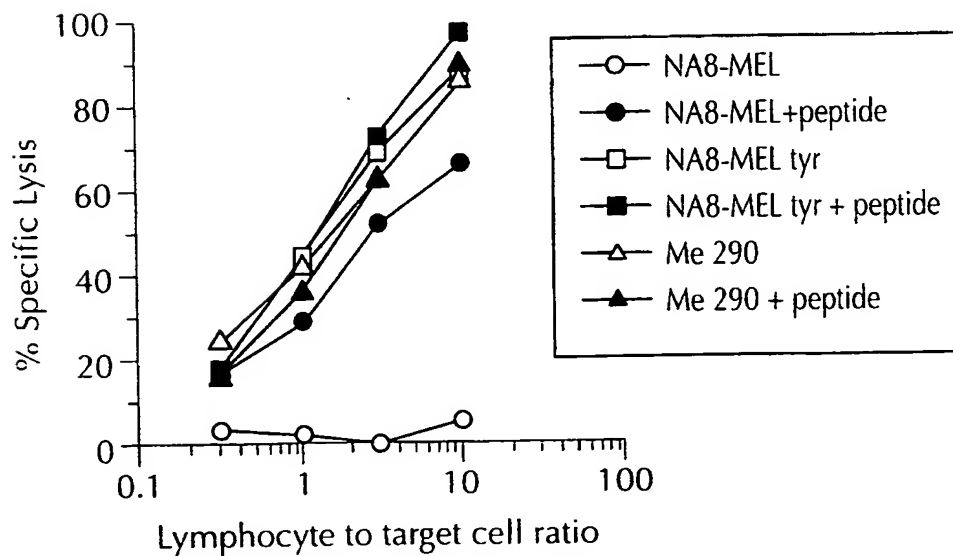
FIG. 5E

FIG. 5F
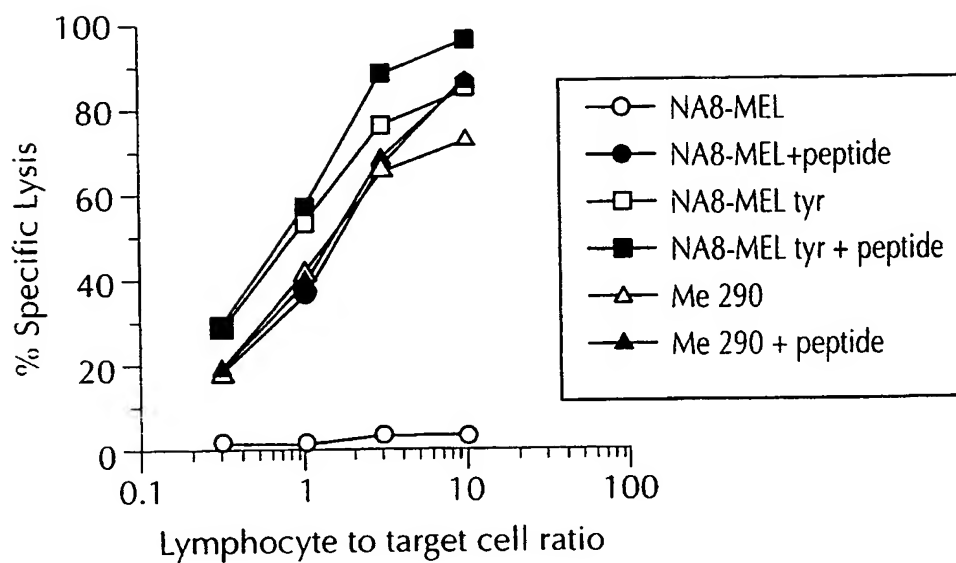
FIG. 5G
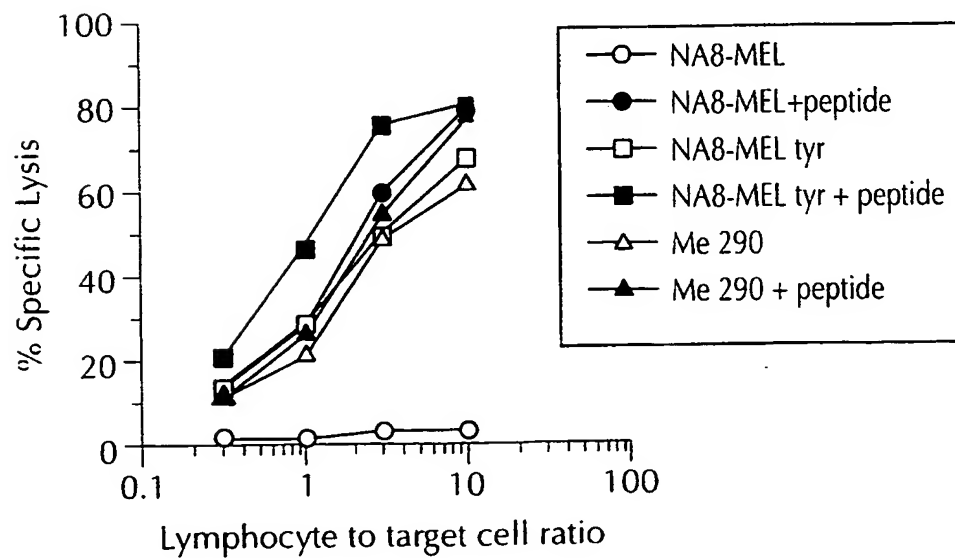
FIG. 5H
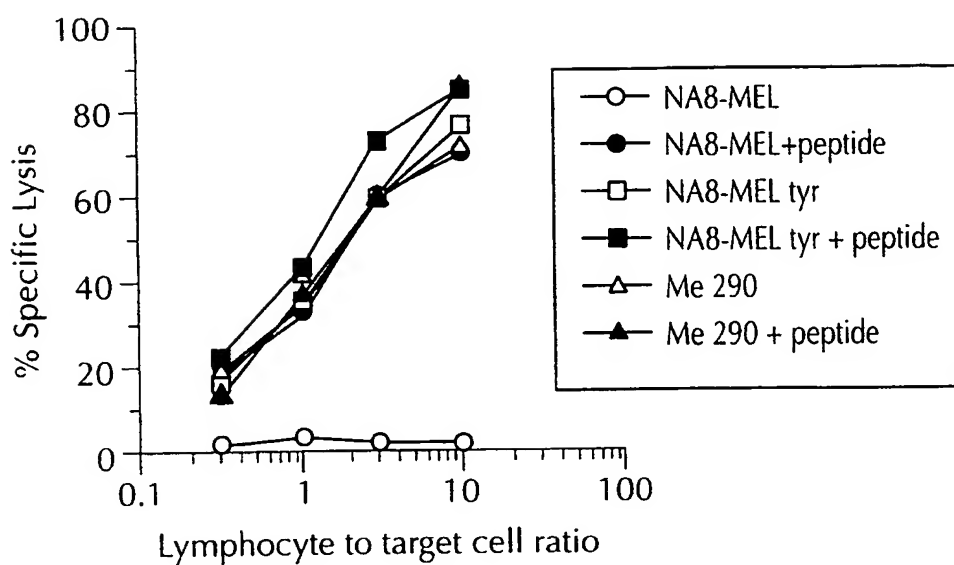
FIG. 6
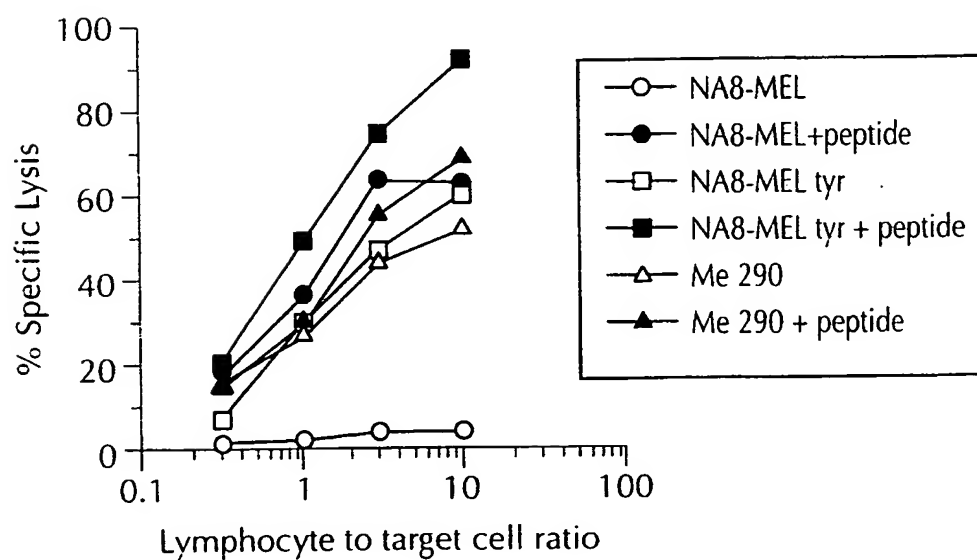
SUBSTITUTE SHEET (RULE 26)

FIG. 7A

FIG. 7B

FIG. 7C

FIG. 8A

SUBSTITUTE SHEET (RULE 26)

FIG. 8B

FIG. 8C

SUBSTITUTE SHEET (RULE 26)

FIG. 8D

FIG. 8E

SUBSTITUTE SHEET (RULE 26)

FIG. 8F

FIG. 9A

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SUBSTITUTE SHEET (RULE 26)

FIG. 9B

% Specific Lysis vs Anti-CD3 antibody (ng/ml)
5:1

FIG. 9C

% Specific Lysis vs Anti-CD3 antibody (ng/ml)
5:1

FIG. 9D

% Specific Lysis vs Anti-CD3 antibody (ng/ml)
30:1